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Microbial metabolites: 45 years of wandering, wondering and discovering

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I would like to dedicate this article to my family and many individuals who have helped and supported me through my scientific endeavors, far too numerous to mention by name here. I would, however, like to single out two people who have provided me with so much more than should be expected during my career, namely Dr. Yukimasa Yagisawa, who helped with crucial guidance, advice, and introductions during my early years and to Professor Max Tishler, who proved to be not just a great teacher and colleague but also a highly respected and greatly valued mentor and true friend

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1. Introduction

This review represents a personal and reflective account of my research and how it has developed over the last 45 years at the Kitasato Institute. At the outset, I would like to acknowledge that my work, the directions it has taken and the results that have been achieved, were greatly influenced by a wide variety of friends and colleagues, too numerous to mention, but to all of whom I am deeply indebted.

My first steps on a lifetime journey in Organic Chemistry began with post-graduate study at the Tokyo University of Sciences. After completion of my studies, I found employment at Yamanashi University where I was able to work on various aspects of the brewing of wines, brandy, and other fermented products. It was at this time that I quickly became fascinated by the seemingly limitless capabilities of microorganisms, which can produce and decompose all manner of compounds. In addition, I had the great good fortune to attend a lecture given by Prof. K. Sakaguchi, who was a pioneer of modern biotechnology in Japan. This lecture still looms large in my memory because it focused on the fact that microorganisms have the capacity to do things and produce things which humans are often unable to do or do not yet know how to do. This fundamental reality has been the driving force of my vision, scientific philosophy, and research ever since.

After 2 years at Yamanashi, in 1965 I was employed by the renowned Kitasato Institute, where the alumni already included some of Japan's—and the world's—greatest microbiologists. I began work studying antibiotics under the supervision of Dr. Toju Hata, who was well known for his discovery of mitomycin. So began my lifetime quest to practically apply my knowledge of organic chemistry and to study, discover, understand, and make use of the vast array of compounds produced by microorganisms, especially those that could be found in soil. Until now, I have spent 45 fulfilling years on this mission, which has been and remains a never-ending journey of discovery, learning, and amazement about how much Nature has to offer us. I have been fortunate to meet with some little success, including identification and elucidation of over 440 new compounds, but still feel as though I have but merely scratched the surface with respect to realizing the promise and potential that microorganisms have to offer.

In the mid-1960s, when I started working on antibiotics, it had already become comparatively difficult to discover new antibiotics and interest in this line of research had begun to wane. My main focus at that time was on determination of the structure of known antibiotics. However, I was concerned that this line of work was relatively easy and offered little future promise and, in particular, offered virtually no opportunities for discovering new compounds. I believed profoundly that we had only just begun to identify the chemicals that microorganisms had to offer and that what was really needed was 'a new way of looking'. Therefore, I turned my attention to finding new antibiotics, or any other biologically active compounds, and to do so by devising novel and innovative screening systems. Fortunately, throughout the following decades, we have been able to discover hundreds of new compounds, most with interesting characteristic with regard to their bioactivity and chemical structure.

It is my belief that we should try and obtain as holistic an understanding as possible of any organism, what it produces, how, the structures involved, and the uses and applications of all such products. Consequently, in this account, an electronphotomicrograph of the producing microorganism is placed alongside the structural formula of each compound. This allows a much fuller understanding of taxonomy as well as recognition of the incomparable contribution of the microorganisms themselves. Indeed, I have made this a custom ever since we began to use a scanning electron microscope (SEM) in the early-1970s, and it is something, which is unique among research groups in the field of microbial chemistry. The scale bar in each SEM image of the producer microorganism corresponds to 1 μm in size, unless otherwise specified.

In this somewhat chronological account of discovery, the total synthesis of the compounds discovered through my research is given, but only in instances where our research group accomplished the first synthesis. Our total synthetic studies were carried out on a limited number of compounds, primarily those that could be obtained only in minute quantities naturally, in order that we could produce enough material for further investigation.

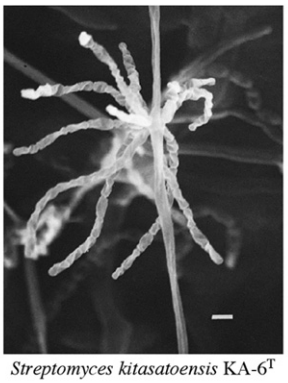
Throughout this review, I will describe a basis for new drug discovery, and detail my approach, which has helped determine how and in what direction those studies have proceeded, which has been published in much greater detail elsewhere.[1,2] Sometimes we were responding to my own vision, sometimes to specific findings by others about a particular disease, cause or biomedical need. I will cite literature that influenced the evolution of our studies. Citations to the findings of others who have worked in these areas are scantily annotated for obvious reasons: more scholarly documentation of these contributions is found in earlier accounts of the work described herein and in the book 'Splendid Gifts from Microorganisms', the fourth edition of which appeared in 2008.[2]

2. The formative years: early studies on antibiotics and the chemical biology of macrolides

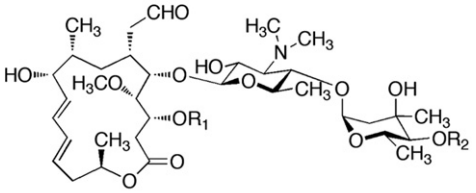
2.1. Structure determination of antibiotics

After arriving at the Kitasato Institute in the mid-1960s, I worked first on the structural determination of leucomycin

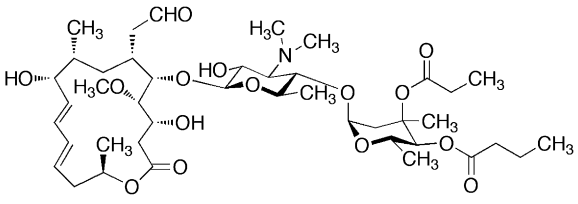
(produced by *Streptomyces kitasatoensis* KA-6^T), which was already being used clinically as a human medicine. I managed to isolate 10 components (A_1, A_3 (**1**), A_4, A_5 (**2**) ~U, V) and determined their chemical structures relatively quickly by making good use of a 60 MHz Nuclear Magnetic Resonance spectrophotometer, which had fortunately been recently installed by the institute (there were only a few such machines in Japan in those days). The structural determination of the leucomycins, 16-membered ring macrolides, represented my entry into research on microbial metabolites. Thereafter, I was able to clarify the absolute structures of josamycin (which is identical with leucomycin A_3 (**1**)), magnamycin, spiramycin (**3**) (see Fig. 22), and tylosin (**4**), as well as the structural relationships of 16-membered ring macrolides that had been discovered at that time due to their relation to leucomycin.[3–5]



Streptomyces kitasatoensis KA-6^T



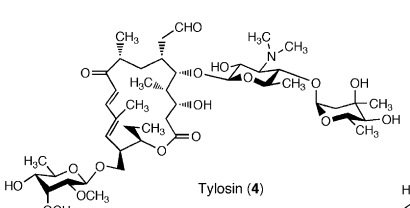
Leucomycin A_1 [R_1 = H; R_2 = $COCH_2CH(CH_3)_2$]
A_3 (**1**) [R_1 = Ac; R_2 = $COCH_2CH(CH_3)_2$] (Josamycin)
A_4 [R_1 = Ac; R_2 = $COCH_2CH_2CH_3$]
A_5 (**2**) [R_1 = H; R_2 = $COCH_2CH_2CH_3$]
A_6 [R_1 = Ac; R_2 = $COCH_2CH_3$]
A_7 [R_1 = H; R_2 = $COCH_2CH_3$]
A_8 [R_1 = R_2 = Ac]
A_9 [R_1 = H; R_2 = Ac]
U [R_1 = Ac; R_2 = H]
V [R_1 = R_2 = H]



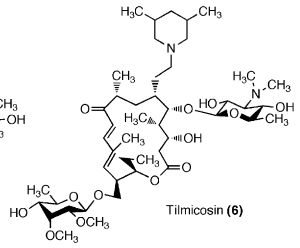
Rokitamycin (**5**)

I recognized early on that collaborations, especially with international partners, greatly facilitated scientific progress. Consequently, I began working with Dr. S. Pestka of the Roche Institute of Molecular Biology, New Jersey, USA, to examine the effect of leucomycins, leucomycin derivatives, and other 16-membered macrolides, such as tylosin and spiramycin, on [^{14}C]-erythromycin binding to *Escherichia coli* ribosomes. The results of these studies enabled the determination of the association and dissociation constant for the binding of each of these macrolides to ribosomes. This led us to the conclusion that the binding to ribosomes in general correlated with their antimicrobial activity, emphasizing my belief that it was important to try and fully comprehend the structure/activity relationship of bioactive compounds.

Soon thereafter, I was instrumental in the development of two semisynthetic antibiotics. Rokitamycin (**5**),[6] a derivative of leucomycin A_5 (**2**), potentiated antibacterial activity and was used for humans, while tilmicosin (**6**),[7] a derivative of tylosin, had practical use in animal health. These successes were achieved though collaboration with Dr. Hideo Sakakibara of Toyo Jozo Co. (now Asahi Kasei Co.) and Dr. Herbert A. Kirst of the Eli Lilly Co. Ltd., respectively. Significantly, tilmicosin still retains an important position as an animal health drug. The collaboration with Toyo Jozo Co. arose as a result of connections established during the days of Dr. Hata, a former president of The Kitasato Institute. In the case of tilmicosin, the research collaboration led to a long-term professional and friendly relationship with the Eli Lilly company and, in particularly with Drs. M. Gorman, R. Hamill and H. Kirst. I am personally convinced that individuals are the true agents of change and that personal friendship, mutual respect, and consideration are essential components for success—in science and for life in general.

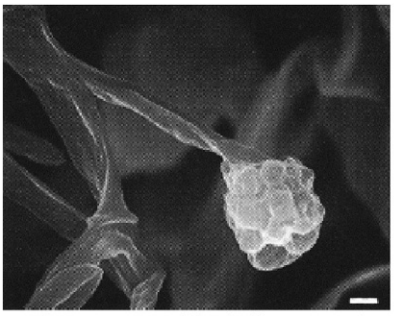


Tylosin (**4**)

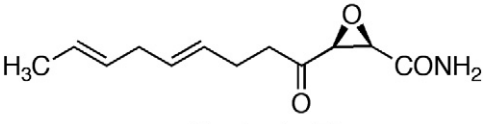


Tilmicosin (**6**)

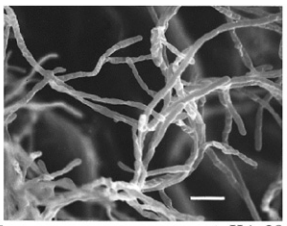
My next focus was on the isolation and structural determination of an antifungal substance, cerulenin (**7**), whose activity had been detected by Dr. Hata and his coworkers in a culture broth of *Cephalosporium* (later renamed *Acremonium*) *caerulens* KF-140.[8] Although the active substance had not yet been isolated, it was named cerulenin. Moreover, I also accomplished the isolation of the kinamycins (A (**8**) ~D) and determined their chemical structures.[9] The nitryl residue in the structural formula of kinamycin A was later corrected to be the azido residue by Dr. S. J. Gould.[10]



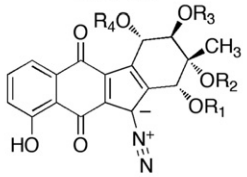
Acremonium (Cephalosporium) caerulens KF-140^T Bar: 5 μm



Cerulenin (**7**)



Streptomyces murayamaensis KA-295
Bar: 5 μm



Kinamycin A (**8**) (R_1 = H; R_2 = R_3 = R_4 = Ac)
B (R_1 = H; R_2 = Ac; R_3 = R_4 = H)
C (R_1 = Ac; R_2 = H; R_3 = R_4 = Ac)
D (R_1 = Ac; R_2 = H; R_3 = Ac; R_4 = H)

Cerulenin (**7**) marked a significant step in my research career. At the time, epoxy squalene was established as a biosynthetic precursor of squalene. Due to the existence of an epoxy and two ethylene residues in the structure of cerulenin, I surmised that it might inhibit sterol biosynthesis, so I discussed this with Dr. S. Nomura at The Kitasato Institute and began to study the action mechanism of the compound. As a result, we found that cerulenin did not inhibit biosynthesis of sterol but rather that of fatty acid.[11] This finding arose shortly before I departed for the USA to take up an invitation from Prof. Max Tishler of Wesleyan University to become a Visiting Professor in his Chemistry Department, newly established after his retirement from his post as head of the Merck Research Laboratories. When I left for the USA in 1971, I took the data and a cerulenin sample with me.

Shortly thereafter, through an introduction by Dr. W. Celmer of Pfizer Co., who had become a close friend and colleague, I met Prof. K. Bloch (Conant Laboratories, Harvard University) and showed him our data, which interested him markedly. Consequently, we started collaborative research using my sample. Within a few months, cerulenin was clarified to be a specific inhibitor of the condensing enzyme (β-ketoacyl ACP synthase), one of the basic enzymes in fatty acid biosynthesis.[12] Fortunately, I was able to learn a great deal about research methods and technology of biochemistry through the collaboration with Prof. Bloch, who had already received the Nobel Prize in 1964. These fundamental insights proved of invaluable use later, particularly with regard to construction of new screening systems.

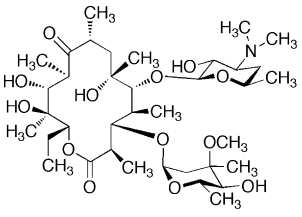
Noticing the results obtained by Prof. Roy Vagelos (Department of Biological Chemistry, Washington University), we began a collaborative study shortly before I returned to Japan in 1973 and found the enzyme to irreversibly inhibit and to be associated with the binding of 1 mol of cerulenin per mole of enzyme.[13] Thus, cerulenin was recognized as the first inhibitor of lipid biosynthesis and can be seen to be the forerunner of all statin-type inhibitors.

Subsequently, we ascertained that cerulenin can inhibit polyketide biosynthesis too. This was the first experimental proof that the biosynthetic mechanisms of fatty acid and polyketides are closely related.[14,15] Cerulenin was notable for its ability to inhibit both biosyntheses of fatty acid and polyketides and so became an important reagent for biochemical research. This was my first compound that became useful as a biochemical reagent, and it has been followed by more than 20 other compounds discovered though screening of novel substances (some examples are shown in Table 3).

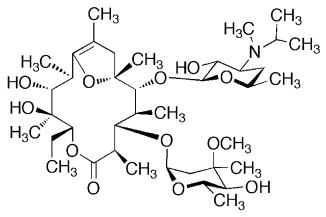
2.2. Chemical and biological studies of macrolides

Widespread studies on the macrolides have confirmed that they exhibit a broad spectrum of bioactivity. In order to study the relationship between antibacterial characteristics, molecular structure, and modes of action, we chemically synthesized various macrolide derivatives and tested for bioactivity.

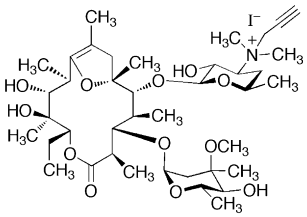
In 1984, Prof. Z. Itoh observed that erythromycin A (EMA) (**9**) promotes contraction of the digestive tract.[16] Subsequently, in collaboration with Prof. Itoh, we obtained erythromycin derivatives (EM574 (**10**) and EM536 (**11**)), which displayed strong gastrointestinal motor stimulating activity but which totally lacked any antibacterial effect.[17,18] Using these compounds, we clarified that the derivatives act as an agonist against motilin, an internal peptide hormone consisting of 22 amino acids. These derivatives, possessing no antibacterial activity but displaying motilin like action, were named 'motilides'.[19] Oddly enough, in 1984, the same year we began work on this topic, Prof. S. Kudoh found that erythromycin A was useful as a remedy for DPB (Diffuse panbronchiolitis).[20]



Erythromycin A (EMA) (**9**)

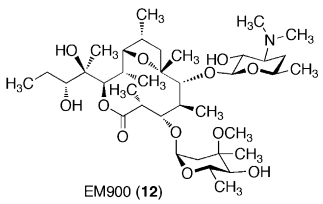


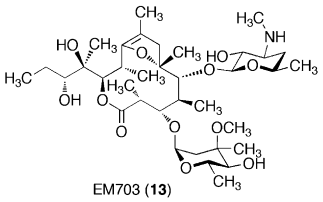
EM574 (**10**)



EM536 (**11**)

Erythomycin (EM) proved to be the mainstay of much of our early work on structure/activity relationships, along with construction of many new compounds. In particular, several novel EM derivatives that have remarkable immunomodulating activities but which have no antibacterial characteristics appeared. We found a new compound, EM900 (**12**), which exhibits better promotive activity of monocyte to macrophage differentiation activity than that of EMA, but without any antibacterial effect.[21] EM900 significantly improves colonic damage in experiments using 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced colitis in rats. The compound also significantly ameliorates cigarette smoke-induced increases in lung functional residual capacity (FRC), residual volume (RV), and alveolar sizes. Taking into account the marked therapeutic effects on experimental chronic inflammatory diseases, antibacterial-free EM900 may be a promising lead therapeutic compound for chronic inflammatory diseases, such as inflammatory bowel disease (IBD) or chronic obstruct pulmonary disease (COPD).[22]

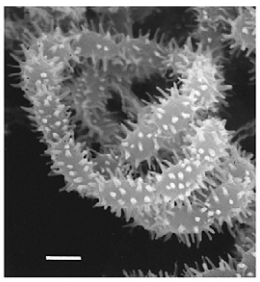

EM900 (**12**)

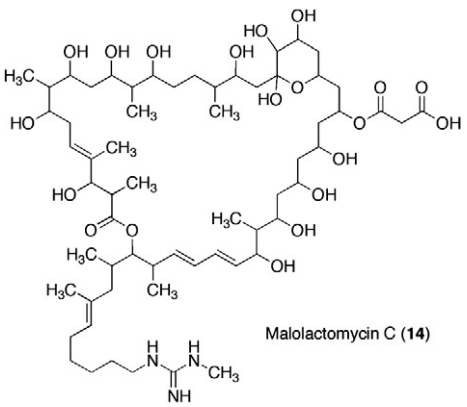

EM703 (**13**)

Recently, through collaboration with Dr. Kiyoko S. Akagawa, we showed that EM 703 (**13**) is a good lead candidate for a drug that can inhibit M-tropic HIV-1 replication in tissue macrophages. This involves a new way of converting their phenotype from HIV-1-susceptible to HIV-1-resistant, through down-regulation of hematopoietic cell kinase (HcK) and the induction of small isoforms of transcription factor CCAAT enhancer binding protein β (c/EBPβ), via modulation of the activation of MAP kinases.[23]

EM has been clinically used since 1953 and so is one of the oldest antibiotics. In the light of recent studies, I expect with certitude the development of more such influential drugs though better elucidation and comprehension of action mechanisms and structure and their interaction in bestowing various biological activities.

My research, which started at the Kitasato Institute with the structural determination of leucomycins, was followed by a continual search for new microbial metabolites. This has resulted in the discovery of many new macrolides, several of which possess interesting and unique bioactivities, such as malolactomycin C (**14**),[24] avermectins (**22**, **23**), virustomycin (**30**), setamycin (**31**), luminamicin (**33**), and phthoramycin (**36**) (as will be described later). It is therefore safe to say that research on macrolides represents the core element of my life's scientific work.


Streptomyces sp. KP-3144


Malolactomycin C (**14**)

3. The discovery years: new screens and novel biologically active microbial metabolites

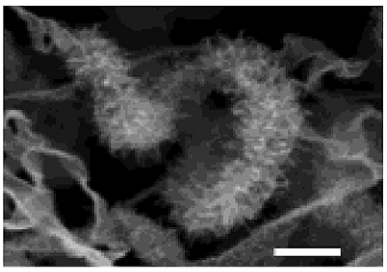
In this section, the fruits of a variety of screening methods are detailed. I have chosen 25 kinds of new screening processes and 37 representative novel compounds they unearthed. This illustrative presentation is in chronological order, with a summary of the key features of the methods, the characteristic metabolites found by each method, the producing microorganisms, chemical structures and their biological properties.

As mentioned, 2 years after entering the Kitasato Institute, I shifted the main focus of my research to the search for new antibiotics and other biologically interesting microbial metabolites. At the time, I was convinced that new and innovative screening systems held the key to the discovery of new compounds—a belief that I have maintained to this day. Through the years, one or two new systems were introduced annually, while older systems were discarded. In general, we now routinely use at least 10 screening systems that we originally devised. New knowledge and understanding, especially in new scientific fields, rarely, appears quickly. Time is an essential ingredient. Time and patience, as well as trial and error, are all basic components in the creation of novel screens. Nevertheless, the undertaking is both pleasant and challenging, much like playing 'Go' or 'Shyogi' (Japanese Chess). A few of our screens have proved successful but have yet to produce the significant results we originally envisaged, although I am of the opinion that it is simply a matter of patiently working out some constraints and refining the screening system and hoping for some good fortune. Indeed, I am constantly reminded of the words of Louis Pasteur; 'Chance favors the prepared mind'. To me this is the only way to approach the study of microorganisms, for we are constantly being faced with new surprises and developments. This is the mindset that I have followed in my quest, and it is one which has allowed Nature to reveal to me well over 440 kinds of bioactive microbial metabolites, several of which have proved of almost immeasurable benefit to humankind, both directly and indirectly.

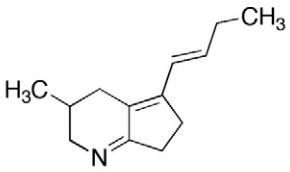
3.1. Chemical screening: the discovery of pyrindicin (15), staurosporine (16), herquline A (18), and other microbial alkaloids

When I joined the Kitasato Institute there were many undergraduate students working under Prof. Hata so, as an Associate Professor, I had to consider research themes for them. In order to advance the search for new antibiotics and other new microbial metabolites, in-depth knowledge and a broad understanding of the Biological Sciences are fundamental requirements. To apply this knowledge to real life situations takes time and so the construction of any screening system is a time-consuming process. At first, we began 'Chemical Screening'. This entailed a search and isolation method to identify and analyze organic compounds in fermentation broths employing a coloring reaction, a basic qualitative analysis of organic compounds. This screening technique commenced about 5 years after I joined the institute. It was based on my profound belief that 'microorganisms never engage in futility, it is just our lack of knowledge and vision that prevents us from understanding'. It proved a great challenge to both isolate microbes and their metabolites and then later assay their biological features. We had to devise new isolation methods as well as new and innovative assaying systems and protocols, but we persevered.

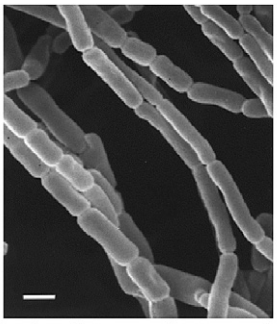
The first compound that we isolated through our new Chemical Screening system was pyrindicin (**15**), which was discovered using Dragendorff's reagent,[25] a coloring reagent of plant alkaloid origin, containing bismuth nitrate and potassium iodide.[26,27] Later, pyrindicin was proved to possess antimicrobial activity.



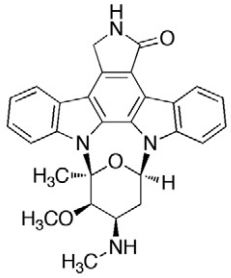
Streptomyces griseoflavus subsp. *pyrindicus* AN-15^T



Pyrindicin (**15**)

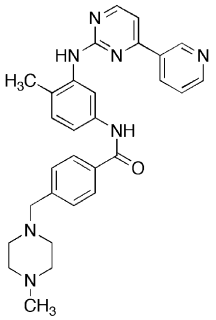


Saccharothrix aerocolonigenes subsp. *staurosporeus* AM-2282^T (*Lentzea albida* AM-2282)



Staurosporine (**16**)

Of far greater significance, the first indolocarbazole compound, staurosporine (**16**), was also found using Dragendorff's reagent.[28,29] Nine years after staurosporine was discovered, Dr. T. Tamaoki et al. found the compound to have potent inhibitory activity against protein kinase C, the first such compound to do so, and so it quickly became one of the world's most prominent research reagents of microbial origin.[30] During the past 35 years, staurosporine and related indolocarbazole natural compounds have been isolated from not only actinomycetes but also myxomycetes (slime molds), cyanobacteria, and marine invertebrates, such as sponges.[31] Staurosporine has been viewed by some as the forerunner of many of the recently introduced anti-cancer agents. I have written recently in detail about staurosporine and related compounds, in a review article with Dr. Nakano,[31] and will not expand on that aspect here. Suffice it to say that several related compounds are being developed as antitumor agents, for example, the development of imatinib (Gleevec) (**17**) has been directed by the biological activity of staurosporine.[32] For me, the discovery of staurosporine was a significant milestone, not just because of its major impact in science and biomedicine, but because it was tangible proof that my beliefs were correct and that microorganisms offer virtually unlimited beneficial products, it is simply a matter of us finding ways to identify and apply them for the good of human society.



Imatinib (Gleevec®) (**17**)

The structure of co-crystal of both staurosporine and cAMP-dependent protein kinase was reported by Dr. D. Bossemeyer (German Cancer Research Centre) et al..[33] Until then, it had only been assumed that staurosporine binds competitively at the ATP-binding site of protein kinase and this was concisely described in their account. This feature of staurosporine is really elegant and artistic, making it a biochemical reagent ideal for use in investigations of signal transduction of cells, which is currently one of the most interesting research fields in biological science. Whenever I look at the complex molecular structure of staurosporine (Fig. 1), it brings a deep feeling of satisfaction and it remains a constant reminder of the validity of our approach to microbial natural-product research. Later, the total synthesis of staurosporine was achieved by the groups of Prof. S. J. Danishefskey et al.[34] and Prof. J. L. Wood et al.[35]

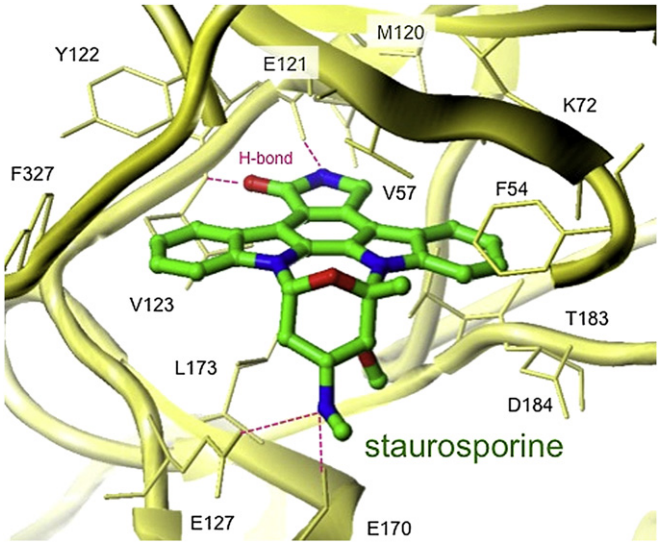
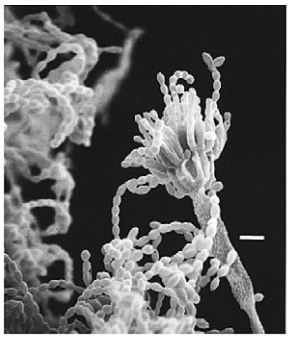
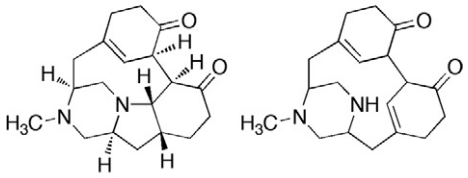


Fig. 1. Complex structure of staurosporine with the catalytic subunit of cAMP-dependent protein kinase.

Our Chemical Screening system cumulatively produced a wide variety of compounds, including the herqulines (A[36] (**18**) and B[37] (**19**)). After discovery, the compounds were found to possess an inhibitory activity for platelet aggregation. At first sight, these compounds look simple yet, to date, no report of their total synthesis has appeared, although many organic chemists have attempted the challenge. It appears that the existence of highly twisted strains of the central nine-membered ring is the major obstacle. For me, this is yet another justification that microorganisms do relatively easily that which we humans find virtually impossible.



Penicillium herquei Fg-372
Bar: 5 μm

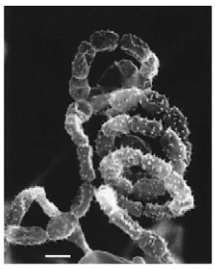


Herquline A (**18**) Herquline B (**19**)

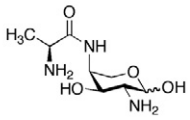
3.2. Avermectins (B_{1a} (22) and B_{1b} (23)), an anthelmintic endectocide

The transcontinental collaboration between my group at the Kitasato Institute, Tokyo and researchers of Merck Sharp and Dohme (MSD) Research Laboratories, in the USA (MSDRL) started in 1973, following my initial approach and with the facilitation of my great friend and mentor Prof. Max Tishler of Wesleyan University, Conn. USA (who was previously head of the Merck Research Laboratories). It resulted in the discovery of an epoch-making compound, avermectin, and its dihydroderivative, ivermectin.

At the beginning of the 1970s, Prof. Yukimasa Yagisawa, General Manager of the Japan Antibiotics Research Association, provided me with highly beneficial advice and guidance and encouraged me to make fullest use of the possibilities that research work overseas could bring to both myself and to Japan. To facilitate this, he used his network of overseas connections and wrote individual letters of introduction for me. As a result, in September 1971, I was granted a sabbatical, which allowed me to take up an invitation from Max Tishler to work as Visiting Professor in his newly-formed Chemistry department at Wesleyan University. There, my initial work focused on the structural analysis of a new antibiotic, prumycin (**20**),[38] which had been found prior to my departure, as well as on the structure/activity relationships of macrolides[39] and the mode of action of cerulenin. The contribution that both of these individuals made to my development, as a scientist, educator, and individual, has been beyond measure.



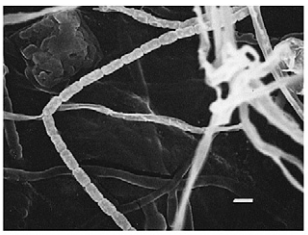
Streptomyces kagawaensis F-1028^T



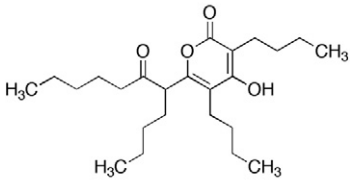
Prumycin (**20**)

It later transpired that I would have to cut short my intended stay in the US to return and take the reins of the Research Department at Kitasato, following the retirement of the current head, and I returned in early-1973. In view of my impending return, and extremely mindful of the critical need to obtain substantial financial resources to fund the research work in Tokyo after I got back, I spent several months visiting many of the major US pharmaceutical companies, pushing my proposal for collaborative research in order to both acquire research funds and to accelerate information exchange. I was greatly encouraged in my mission as every company I approached expressed interest in my proposal. Eventually, I decided to collaborate with the MSDRL, primarily because of Max Tishler's close connection with Merck and his personal linkage to Dr. L. H. Sarrett, then-President of MSDRL. Immediately after I returned to Tokyo, we concluded the agreement for the collaborative research, which started in April 1973. Initially, the main goal was to find growth promoting antibiotics suitable for animals, enzyme inhibitors and general purpose antibiotics produced by microorganisms, but the work soon expanded to encompass other targets.

As a result of the collaboration, a variety of compounds were discovered, boasting a range of interesting biological activities and structures. We found several different kinds of compounds, for example, vineomycins A_1 (**26**) and B_2 (**27**)[40] (see Section 3.3) and setamycin (**31**)[41] (see Section 3.6), both of which have unique structures; aurantinin A (**111**)[42] (see Section 4.1), which was the first polyketide antibiotic of bacterial origin; and elasnin (**21**),[43] which represented the first human elastase inhibitor of microbial origin.



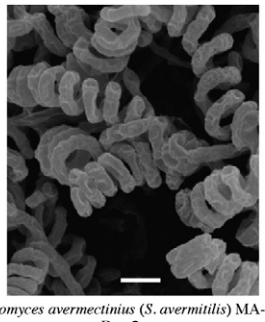
Streptomyces noboritoensis KM-2753



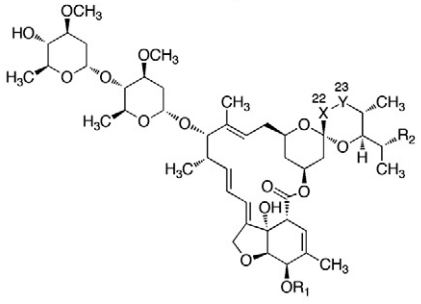
Elasnin (**21**)

Of undeniably the greatest importance, however, was the discovery of avermectin, an anthelmintic antibiotic, and the world's first 'endectocide'. Indeed, the term was introduced simply to identify this

unique compound. Simply put, avermectin proved to be one of the world's most remarkable biomedical discoveries, being accompanied by a number of international 'firsts' and having immeasurably beneficial impact on animal and human health around the world. As the basis of the collaborative research program, our group carried out isolation of microorganisms, culturing and then in vitro evaluation of the bioactivity of any compounds we deemed to be of potential interest. The MSDRL team evaluated all the samples that we sent them in vivo. As MSDRL began to widen their screening systems and introduce new screening models, a decision was taken by Dr. Sarrett to begin screening complete fermentation broths that were to be processed, identified as promising and supplied by Kitasato. From one culture sent by us and using an innovatory *Nematospiroides dubius*-infected mouse screen,[44,45] Dr. W. Campbell et al. found an actinomycete, strain MA-4680, which produced a compound possessing excellent antiparasitic activity. The producing microorganism and its active product were named *Streptomyces avermitilis* MA-4680 and avermectin, respectively, and the structure of the compound was also elucidated.[46] The strain was later renamed *Streptomyces avermectinius*.[47] Researchers at MSDRL continued the screening intensively thereafter—as have many others over the ensuing decades—but no other avermectin-producing organism has ever been found. The strain that we isolated from soil in Japan remains the only avermectin-producing organism ever found. Fortunately, this at-the-time unique collaboration between a Japanese public-sector institute and a US-based private-sector pharmaceutical giant operated under a mutually respectful and altruistic scientific basis. Of note, Dr. Boyd Woodruff was delegated from MSDRL to work directly and closely with our researchers in Tokyo and I think his personal contribution and mediation was a significant factor in making the collaboration such a great success.



Streptomyces avermectinius (*S. avermitilis*) MA-4680^{T}
Bar: 2 μm



Avermectin A_{1a} [R_1 = CH_3; R_2 = C_2H_5; X-Y = ⌇⌇]
A_{1b} [R_1 = R_2 = CH_3; X-Y = ⌇⌇]
A_{2a} [R_1 = CH_3; R_2 = C_2H_5; X-Y = CH_2-CH(α-OH)]
A_{2b} [R_1 = R_2 = CH_3; X-Y = CH_2-CH(α-OH)]
B_{1a} (**22**) [R_1 = H; R_2 = C_2H_5; X-Y = ⌇⌇]
B_{1b} (**23**) [R_1 = H; R_2 = CH_3; X-Y = ⌇⌇]
B_{2a} [R_1 = H; R_2 = C_2H_5; X-Y = CH_2-CH(α-OH)]
B_{2b} [R_1 = H; R_2 = CH_3; X-Y = CH_2-CH(α-OH)]

Ivermectin: mixture of B_{1a} (**24**) [R_1 = H; R_2 = C_2H_5; X-Y = CH_2-CH_2] and
B_{1b} (**25**) [R_1 = H; R_2 = CH_3; X-Y = CH_2-CH_2]

Not surprisingly, there has been a wide variety of publications dealing with the avermectin/ivermectin story[48,49,50] including recent articles,[51,52] so I will only summarize very briefly in this account. It is known that avermectin is composed of eight key components. Among them, the mixture of dihydroderivatives at positions 22 and 23 of components B_{1a} (**22**) and B_{1b} (**23**) created the compound called ivermectin (**24**, **25**).[53] Ivermectin proved to be very safe and highly effective and, consequently, was put on the Animal Health market as an anthelmintic agent in 1981. Two years later, it became the biggest selling 'Blockbuster' drug in animal health, a position it has maintained for a quarter of a century, being used to treat billions of commercial livestock and companion animals around the world. Avermectin products have also been used as insecticides in commercial agriculture and for gardening use.[50]

Thanks to the vision of Merck scientists and the commitment of a unique and far-reaching group of international partners, ivermectin has had—and continues to have—a quite remarkable impact on human health—one that myself and my colleagues involved in the early days of discovery could never have imagined.

Another of the global 'firsts' associated with avermectin/ivermectin, was that, in 1987, ivermectin was donated free of charge by the manufacturer (Merck & Co. Inc.) for the treatment of Onchocerciasis (River Blindness) in humans, a disease, which had long brought gross disfigurement, debility, and socioeconomic devastation to millions of the world's poorest people. Ivermectin proved to be an extremely safe, extremely effective cure for River Blindness and the donation was for as long as the drug was required, in the amounts that were needed. This was the first such large-scale drug donation initiative and it has resulted in the world's largest, longest-running and most successful donation program—one that has proved a model for many subsequent donations. Thanks to the gift of ivermectin, the global elimination programs for two of the world's most disfiguring and socioeconomically crippling tropical diseases, namely River Blindness and Lymphatic filariasis (or Elephantiasis) are swiftly moving towards accomplishing their goals. Goals are being achieved solely or primarily due to the mass community administration of ivermectin.

Onchocerciasis is caused by a nematode, *Onchocerca volvulus*, which lives for 14–15 years in the human body, female worms continually producing several millions of microfilaria during their lifetime, with the worms being transmitted to humans via the bite of a blood-feeding blackfly. Ivermectin only kills immature worms, so entire communities infected have to take ivermectin annually for up to 15 years, until the adult female worms die naturally. Carcasses of dead worms cause potentially severe immune reactions, particularly damaging in the skin and eyes, leading to loss of sight and terrible, incessant itching and destruction of healthy skin. The disease is found primarily in 30 countries in sub-Saharan tropical Africa, with 120 million people still threatened by the disease.

Another tropical disease, Lymphatic filariasis, caused by infection with the filarial worms *Wuchereria bancrofti* and *Brugia malayi or Brugia timori* is even more widespread, disfiguring and stigmatizing. It is estimated that there are 120 million people throughout tropical and subtropical areas infected in some 83 countries in Africa, the Americas and, predominantly, in Southeast Asia. Following a World Health Assembly resolution in 1997, which identified the disease as being one that could be eliminated, and following the extension of the ivermectin donation program to cover Lymphatic filariasis where it co-exists with onchocerciasis, the disease is slated for elimination by 2020.

The sheer scale of these disease elimination enterprises is staggering. Today, some 200 million people a year, mostly among the world's poorest and most disadvantaged communities, are taking ivermectin tablets. Besides being the sole or primary tool in the two global disease elimination programs, ivermectin is being used ever more widely as a remedy for strongyloidiasis (which afflicts 35 million people annually) and to treat and prevent scabies (of which 300 million cases are reported each year). Each year, more uses for the avermectins, and ivermectin in particular, are being found in human and animal health as well as 'off-label' uses.[52]

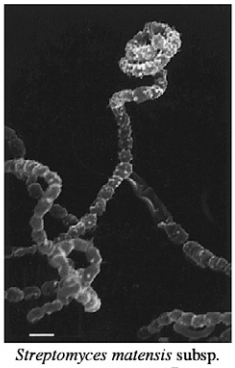
The discovery of avermectin has contributed greatly to improve lives and living standards of billions of people around the world, as well as to improve the health of hundreds of billions of livestock and pets. Development, donation, and distribution of the drug have been associated with many highly beneficial precedents. The substantial royalties earned by the Kitasato Institute on sales of ivermectin in Animal Health have also been used wisely and beneficently. They have funded a great deal of highly-focused research, been used to obtain the land covering 27 ha at Kitamoto City in Saitama Prefecture and to construct a 440-bed district general hospital and a nursing college. At present, over 1000 patients per day visit the hospital, which covers a catchment area that was previously grossly underserved with medical facilities.

Ivermectin is indisputably a 'Splendid gift from microorganisms' and it has bestowed its greatest influence on the world's most poor and disadvantaged. We placed a ceramic plate of a scanning electron micrograph of *S. avermectinius* at the entrance hall of the Kitamoto hospital to illustrate the true foundations on which the building has been constructed and to remind us all of the bounty that still lies hidden in soil, in Japan and elsewhere, awaiting discovery.

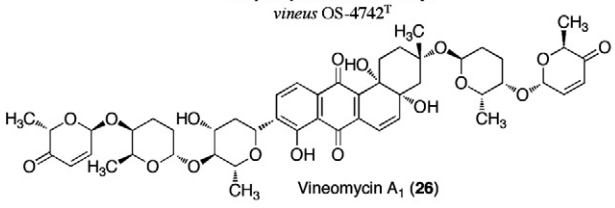
With such an important discovery, it is little wonder that our group has worked long and hard to study every aspect of this particular compound, its biosynthesis and its properties. Our extensive and intensive work in this respect is detailed in separate Sections 4.1–4.4. With regard to the first total synthesis of avermectin B1a,[54] this was achieved by Prof. S. Hanessian and later by Prof. S. V. Ley (the 2009 Tetrahedron Prize winner) and Prof. J. D. White's team. Prof. S. J. Danishefsky (the 1996 Tetrahedron Prize winner) et al. have also reported that they have accomplished the total synthesis of avermectin A1a.[55]

3.3. Vineomycins A_1 (26) and B_2 (27), inhibitors of collagen prolyl hydroxylase

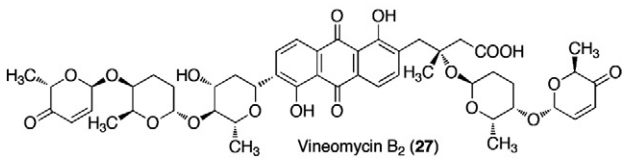
While undertaking general screening for new antibiotics from actinomycetes, vineomycins A_1 (**26**) and B_2 (**27**) were isolated from the culture broth of *Streptomyces matensis* strain OS-4742^T. These compounds are active against Gram-positive bacteria and the Sarcoma 180 solid tumor in mice.[40,56]



Streptomyces matensis subsp. *vineus* OS-4742^T



Vineomycin A_1 (**26**)



Vineomycin B_2 (**27**)

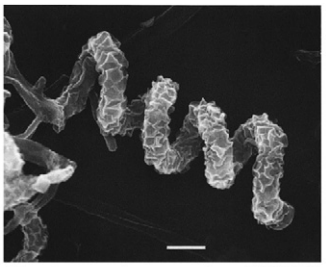
Later, vineomycin A_1 was found to possess potent inhibitory activity against collagen prolyl hydroxylase.

Danishefsky et al. reported the first total synthesis of vineomycin B_2 methylester.[57] Since then, the total synthesis of vineomycin has been reported by many groups, including that of Dr. M. A. Tius.[58]

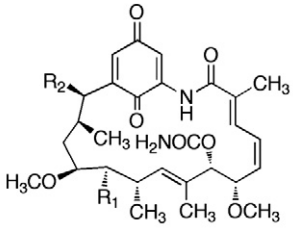
3.4. Herbimycin A (28); an Hsp-90 inhibitor

Herbimycins A (**28**), B, and C, were found through screening designed to identify herbicidal activity.[59,60] The structures of the compounds were confirmed by X-ray crystallographic analysis.[61]

After Y. Uehara et al. reported herbimycin A to be a specific inhibitor of p60src-associated protein kinase, the compound was rapidly and vigorously investigated as a potential antitumor agent.[62] Now, 17-AGG, a related compound, is being developed as an anti-cancer compound. This research is directly connected to the discovery of the specific inhibitory activity of cytosolic chaperone Hsp-90 by W. B. Pratt.[63,64] Prof. K. Tatsuta accomplished the first total synthesis of herbimycin A.[65]



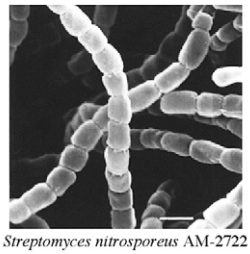
Streptomyces hygroscopicus AM-3672



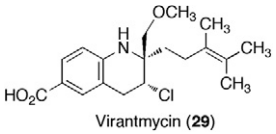
Herbimycin A (**28**) (R_1 = OCH_3; R_2 = OCH_3)
B (R_1 = OH; R_2 = H)
C (R_1 = OH; R_2 = OCH_3)

3.5. Virantmycin (29) and virustomycin A (30); antiviral antibiotics

Virantmycin (**29**) was isolated from the culture broth of *Streptomyces nitrosporeus* strain AM-2722 and identified as an antiviral compound by the plaque reduction method.[66–68] The absolute configuration of virantmycin was elucidated by asymmetric total synthesis of antipode of virantmycin (29).[69] The first total synthesis was reported by Drs. Raphael and Hill.[70]

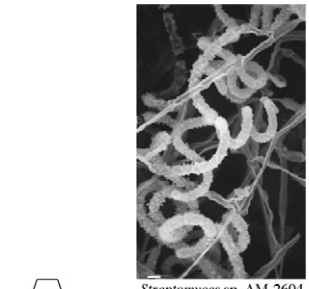


Streptomyces nitrosporeus AM-2722



Virantmycin (**29**)

Virustomycin A (**30**), another antiviral agent, which contains an 18-membered ring lactone structure, was isolated from *Streptomyces* sp. AM-2604 by the same process.[71,72] It is capable of halving plaque formation by both RNA and DNA viruses at a very low concentration (3 ng/ml).



Virustomycin A (**30**)

3.6. Setamycin (31); an inhibitor of V-ATPase

Setamycin (**31**), discovered during regular standard screening for antibacterials, was found to have antitrichomonas and nematocidal properties.[41,73]

Setamycin (**31**)

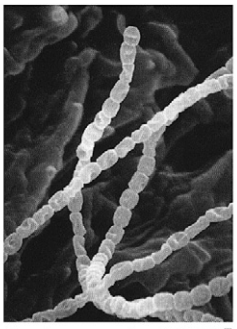
Three years after its discovery, G. Werner et al. isolated the same compound and named it bafilomycin B1.[74] E. J. Bowman et al. then found that the compound specifically inhibits the V-ATPase which controls pH in lysosomes (Fig. 2).[75] The structure of the V-ATPase which is the target of setamycin reported by M. Forgac is shown in Fig. 2.[76] Consequently, this compound has become widely employed as a versatile biochemical reagent owing to its highly specialized activity.

Based on concise taxonomic studies of the producing organism by Prof. Y. Takahashi of our research group, we demonstrated that the organism represented a new genus. As the soil sample was collected at a location near to my home (in Seta, Setagaya-ku, Tokyo) and in view of the august name of our home institute, namely Kitasato, the new organism was named *Kitasatospora setae*.[77]

This event marked the first of our discoveries of new genera of microorganisms. Since that initial step, we have so far found 9 new genera and over 30 new species during the course of our research (Table 2).

3.7. Cervinomycin A_1 (32) and luminamicin (33); antianaerobic bacteriacides

On the basis of the idea that antibiotics effective for intestinal bacteria will be advantageous as growth promotors in animal husbandry, we screened for compounds active against anaerobic bacteria and discovered several new compounds having interesting structures and bioactivities. Cervinomycin A_1 (**32**) was first discovered as an antibiotic active against *Mycoplasma pneumoniae*, a bacterium causing pneumonia in humans. Further testing proved that the compound had more potent activity against other problematic anaerobic bacteria, such as *Clostridium perfringens*.[78] T. R. Kelly et al. achieved the first total synthesis of cervinomycin A_1 (**32**).[79]



Cervinomycin A_1 (**32**)

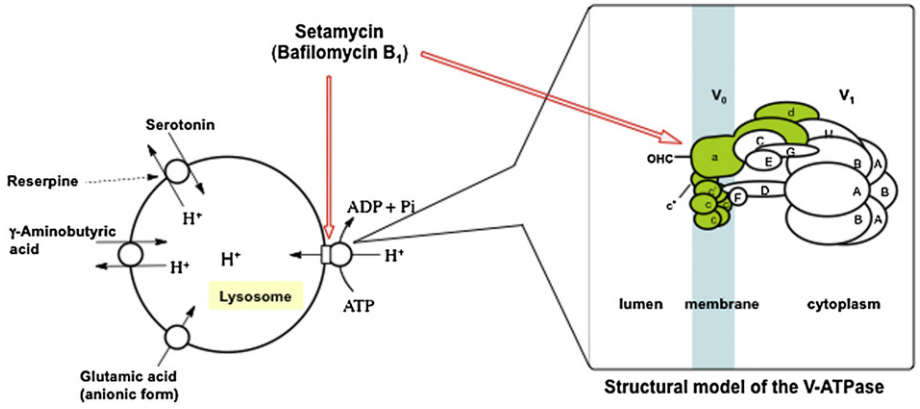
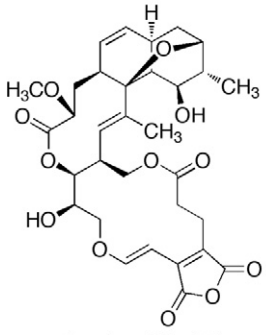


Fig. 2. Inhibitory mechanism of V-ATPase by setamycin (**31**) (bafilomycin B1).

In contrast, luminamicin (**33**) was found during screening for specific inhibitors of *Clostridium perfringens* growth, and it proved to selectively prohibit growth in several other key anaerobic bacteria, such as *Clostridium difficile*, as well.[80]

The absolute structure of luminamicin was determined using conformational analysis via high-temperature molecular dynamics, NMR spectroscopy, and the modified Mosher method.[81] The 3-dimensional structure of luminamicin shows an interesting feature in that the maleic anhydride functionality, in conjugation with the enol ether group of the 14-membered ring macrolactone, is nearly perpendicular to the plane of the other two rings.

Streptomyces sp. OMR-59



Luminamicin (**33**)

3.8. Phosalacine (34), oxetin (35), phthoramycin (36), and phthoxazolin A (37); microbial metabolites possessing herbicidal activity

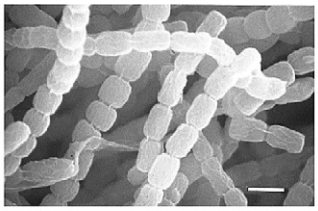
Herbicidal agents are vitally important for agriculture and agribusiness and, as ecological knowledge and awareness has improved over the years, the necessity of finding 'environmentally-friendly' compounds has become paramount.

Virtually from the outset, we introduced a screening system constructed to identify compounds which had high specificity on vegetative growth. One of our first targets in this respect was screening for glutamine-competitive metabolites.

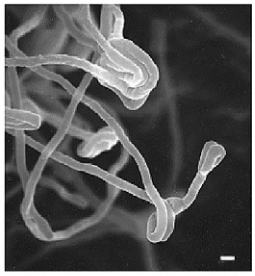
Plants generate ammonia when metabolizing nitrogenous fertilizers, with the resulting ammonia being toxic for most plants. Glutamine synthetase in plants renders the ammonia non-toxic by binding it to glutamic acid. Moreover, the amide nitrogen of the resulting glutamine has a secondary use, being exploited as a nitrogen source in various other metabolic pathways. Consequently, we opted to screen for metabolites which were glutamine competitive and which might disrupt the enzyme, eventually finding phosalacine (**34**), which demonstrates potent herbicidal activity.[82] The structure of phosalacine is similar to that of bialaphos, in which L-Leu is replaced with L-Ala at the carboxyl terminal, with the latter already being used in commercial agricultural products.

A novel amino acid, oxetin (**35**), was also discovered in the same screening system.[83] Oxetin shows herbicidal activity and was found to exert this effect by inhibiting glutamine synthetase.

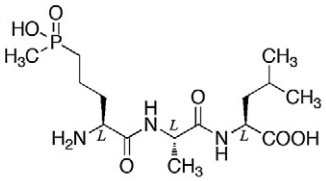
The total synthesis of oxetin was achieved by collaborative research between our group and that of Dr. N. Ikekawa.[84]



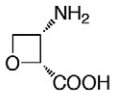
Kitasatospora phosalacinea KA-338^T



Streptomyces sp. OM-2317



Phosalacine (**34**)



Oxetin (**35**)

Cellulose biosynthesis is essential for the growth of plants, so we devised a screen to identify herbicidal activity arising through the inhibition of this vital process. Our screen was established based on the selective antimicrobial activity of actinomycete cultures against *Phytophthora nicotianae* var. *nicotianae* (Fig. 3), a phytopathogenic fungus known to contain cellulose as one of the essential cell wall constituents. Promising samples were then tested against common fungi, such as *Candida albicans* and *Piricularia oryzae*, which do not contain cellulose in their cell walls. As a result, we discovered phthoramycin (**36**) and phthoxazolin A (**37**) from actinomycetes.[85,86] As shown in Fig. 4, phthoxazolin A was confirmed to possess herbicidal activity. It also inhibits cellulose synthesis in cell-free and resting cell systems in *Acetobacter xylinum*, whose cellulose synthesizing machinery resembles that of plants. The first total synthesis of phthoxazolin A was achieved by A. Whiting et al.[87]

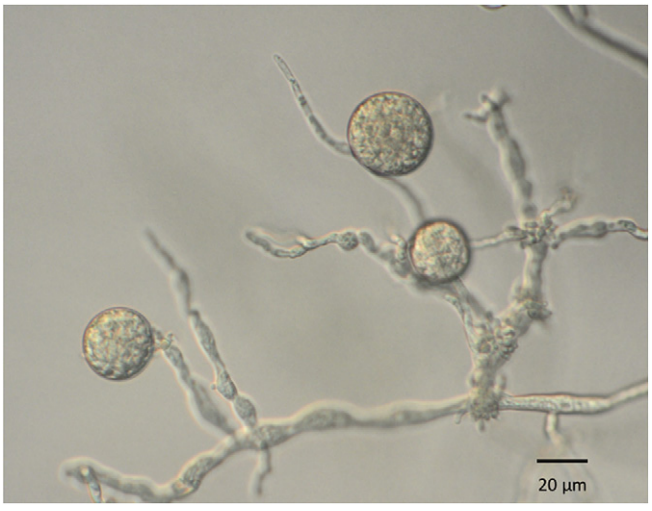
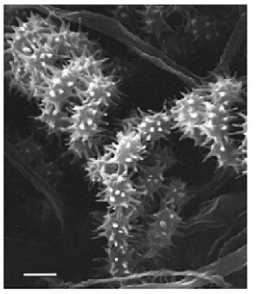
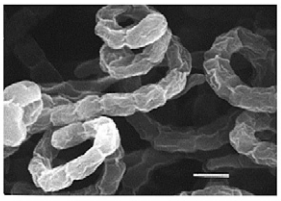


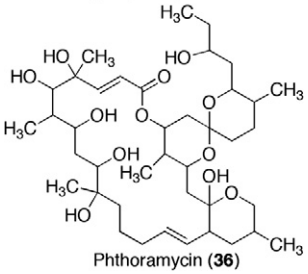
Fig. 3. Electron micrograph of *Phytophthora nicotianae* var. *nicotianae* KF265 (NBRC 4873).

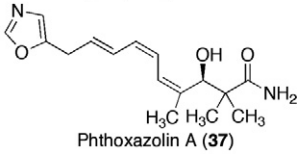
Fig. 4. Herbicidal activity of phthoxazolin A against Velvetleaf (*Abutilon theophrasti*) on post-emergence treatment.

As described later, triacsin C (**41**), lactacystin (**47**), macrosphelide A (**64**), madindoline (**68**), and guadinomine A (**76**) were discovered through a screening system using special cells or mutant bacteria.[88] This originality and innovatory screening processes to identify microbial metabolites is the critical backbone of our work and is a signature of our research.


Streptomyces sp. WK-1875


Streptomyces sp. OM-5714


Phthoramycin (**36**)


Phthoxazolin A (**37**)

3.9. Diazaquinomycins A (38) and B (39); antifolate antibiotics

Diazaquinomycins A (**38**) and B (**39**) were discovered following introduction of a new screening system for antifolates.[89–92] Most microorganisms cannot incorporate folate-related compounds but for some microorganisms, such as *Streptococcus* sp. and *Lactobacillus*, folate-related compounds are essential. We therefore isolated metabolites showing inhibitory activity against a *Streptococcus* sp. grown in a medium containing a limited amount of pteroate, amino acids, nucleic acid bases and nucleosides (except thymine and thymidine (TdR)), but which lacked activity against organisms grown in the same medium supplemented with a sufficient amount of TdR.

The inhibitory site of diazaquinomycin A was confirmed to be thymidylate synthase (Fig. 5), the compound competitively inhibiting

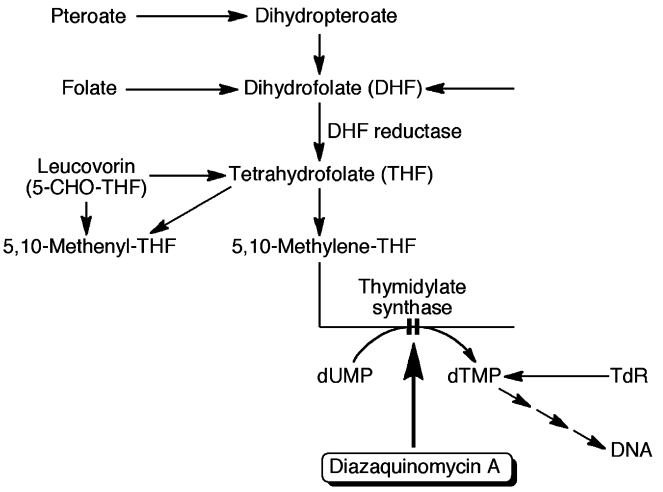
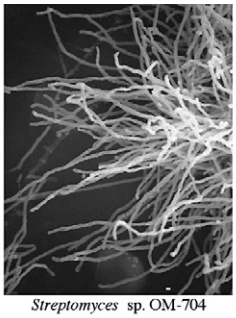
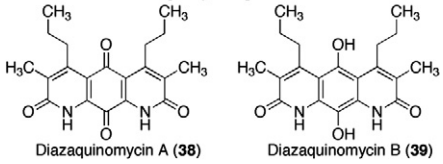


Fig. 5. The metabolism of folate and its related compounds and targets of diazaquinomycin A (**38**).

bacterial and mammalian thymidylate synthases. Diazaquinomycin A inhibits the growth of Gram (+)-bacteria ad Meth-A fibrosarcoma.

Dr. T. R. Kelly et al. reported the first total synthesis of these compounds.[93]


Streptomyces sp. OM-704


Diazaquinomycin A (**38**) Diazaquinomycin B (**39**)

3.10. Triacsin C (41); an inhibitor of acyl-CoA synthetase

Inhibitors of fatty acid metabolism were sought using an assay system employing two mutants of *Candida lipolytica* (Fig. 6), one an acyl-CoA synthetase I (ACS-I)-deficient (L-7) organism, the other a fatty acid synthetase (FAS)-deficient (A-1) mutant. Triacsins A (**40**), C (**41**), and D were found and all inhibited the growth of mutant A-1, but not mutant L-7 on a common medium supplemented with fatty acid and glucose, respectively.[94] Later, it was found that triacsin C (**41**), which is identical with WS-1228A originally isolated as a vasodilator,[95] specifically inhibits long chain acyl-CoA synthetase but not short chain acy-CoA synthetase.[96] This compound is lethal to animal cells, but not to microorganisms,[97] probably due to the different end products (free fatty acid or acyl-CoA) produced by fatty acid synthetases. Triacsin C inhibited macrophage-derived foam cell formation completely by depleting the acyl-CoA required for synthesizing cholesteryl esters (CE) and triacylglycerols (TG).[98]

In this account two inhibitors of lipid metabolism, cerulenin and triacsin C, are covered. The full range of metabolites with inhibitory activity against lipid metabolism discovered by my research group has been described elsewhere.[99]

The first total synthesis of triacsin C (WS-1228A), the most potent of the tricasins, was reported by Dr. H. Tanaka et al.[100]

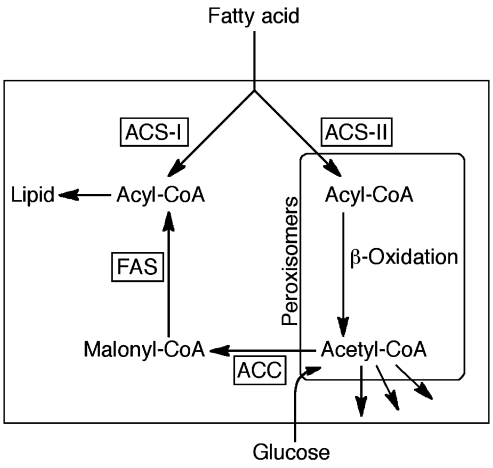
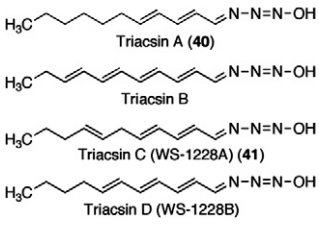


Fig. 6. Fatty acid metabolism of *C. lipolytica*.

Streptomyces sp. SK-1894

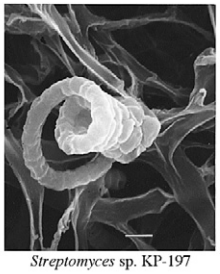


3.11. Jietacin A (42); a nematocide

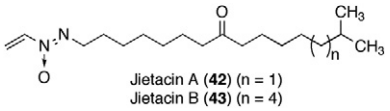
In the 1960s, when the Pine trees which formed a significant part of Japan's unique and beautiful scenery began to die en masse, scientists turned their considerable attention to determining the cause for the die off and to find a solution. Following our discovery of avermectin in the early-1970s, we investigated the nematocidal effect of the drug against the pathogenic nematode, *Bursaphelenchus lignicolus*, which had been identified as the source of the Pine tree problem. Unfortunately, we could not find any effect in vivo, but did so in vitro. So we began to use this process to screen for nematocidal activity.

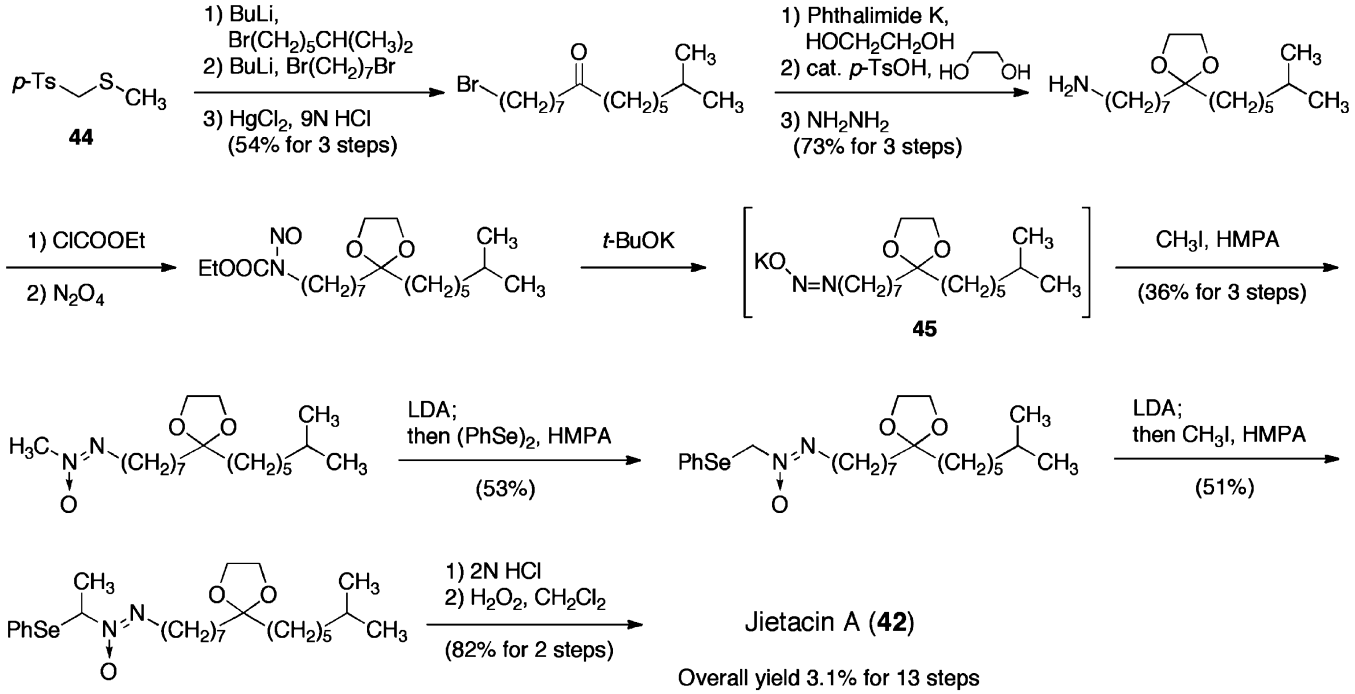
Jietacins A (**42**) and B (**43**) were found to be produced from *Streptomyces* sp. using the new *B. lignicolus* screen.[101] The structure of these compounds contains unique vinylazoxy moieties.[102] They showed 10-fold higher nematocidal activity than avermectin B_{1a} (**22**) against *B. lignicolus* I in vitro.[101]

We accomplished the total synthesis of jietacin A via a concise and efficient route through construction of the alkyl side chain using (methylthio) methyl sulfone (**44**), followed by formation of the azoxy moiety via a regioselective alkylation of diazoate (**45**) (Scheme 1).[103] This method also allowed the preparation of a variety of analogs, and field trials of jietacins are under way using synthetic material.



Streptomyces sp. KP-197

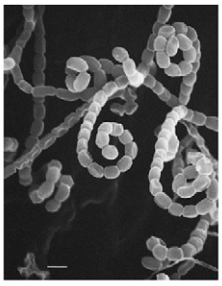




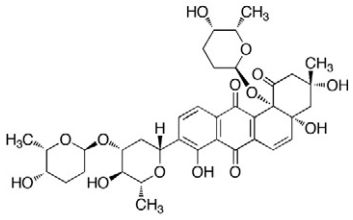
Scheme 1. Total sythesis of jietacin A (**42**).

3.12. Aggreticin (46); an inhibitor of platelet aggregation

The isotetracenone antibiotic, aggreticin (**46**), was identified through a screening system designed to identify inhibitors of platelet aggregation.[104] Aggreticin significantly inhibited the aggregation of rabbit blood platelets induced by ADP, arachidonic acid and PAF (platelet activating factor). However, no inhibition was observed with collagen-induced aggregation.



Streptomyces sp. OM-4842



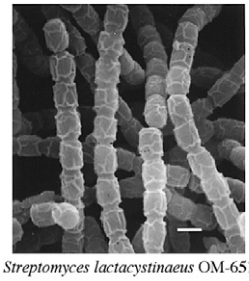
Aggreticin (**46**)

3.13. Lactacystin (47); an inhibitor of proteasomes

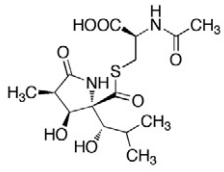
Following addition of β-NGF, a hormone like protein consisting of 118 amino acids, into tissue cultures of nerve cells, the cells differentiate and induce nerve cell-specific neurites. To follow this up, we devised a screen for substances possessing such an activity using Neuro 2a, a cell line of murine neuroblastoma cells, and discovered a compound named lactacystin (**47**).[105] This initiative was very significant for us, as it represented out first foray into screening for bioactive compounds using specific animal cells. The absolute structure of lactacystin was confirmed by X-ray crystallography analysis.

Lactacystin was found to be an inhibitor of proteasomes by G. Fenteany et al.[106] resulting in it being widely used for studies of proteasomes, as well as being used for the elucidation of enzyme function. The work of E. J. Corey found that lactacystin transforms non-enzymatically to a β-lactone derivative, named omuralide (**48**) (see Fig. 7), and the resulting compound acts on proteasomes.[107,108] The molecular structure of rat liver 26S proteasome, based on electron micrography reported by K. Tanaka, is shown in Fig. 7.[109] After clarifying the mode of action of lactacystin, it was reported that it did not mimic any nerve growth factor.[108] However, this conclusion was contrary to the evidence provided through the monitoring of neurite outgrowth of Neuro-2a cells, the basis of the screening by which lactaystin was discovered, a. discrepancy which remains to be resolved.

At present, an increasing variety of new and specialized animal cell lines are becoming available, so I think that novel and bioactive compounds having various activities can be found, if some these new cell lines are used to create new screening systems.



Streptomyces lactacystinaeus OM-6519^T



Lactacystin (**47**)

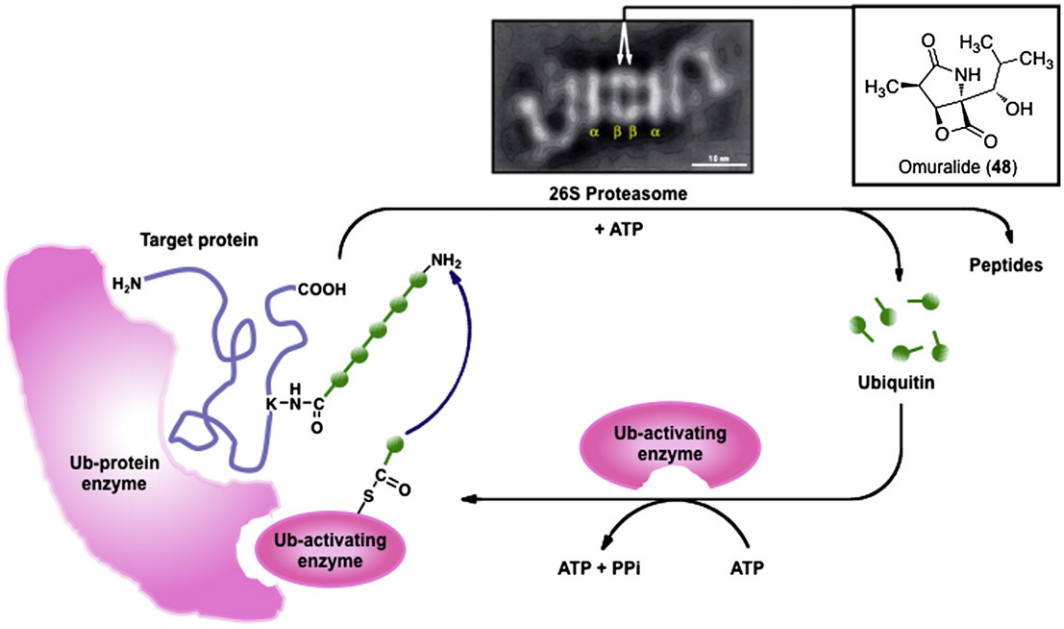


Fig. 7. Model of intracellular protein degradation mediated by the ubiquitin-protease system.

3.14. Pepticinnamin E (49) and andrastins A (50)~D; inhibitors of protein farnesyltransferase

Post-translational modification of *ras* proteins at the specific carboxy-terminal is required for the proteins to localize in the inner side of the plasma membrane and to exert transformational activity. The process of post transylational modification of *ras* p21 is shown in Fig. 8. After farnesylation of cys 186 of *ras* p21, by protein farnesyltransferase, three step reactions (namely proteolysis of C-terminal amino acids, methylation of the terminal carboxyl group and palmitoylation of cysteine) take place to induce malignant transformation. Inhibition of, for example, isoprenylation, should alter membrane localization and the transforming nature of the *ras* oncogene.

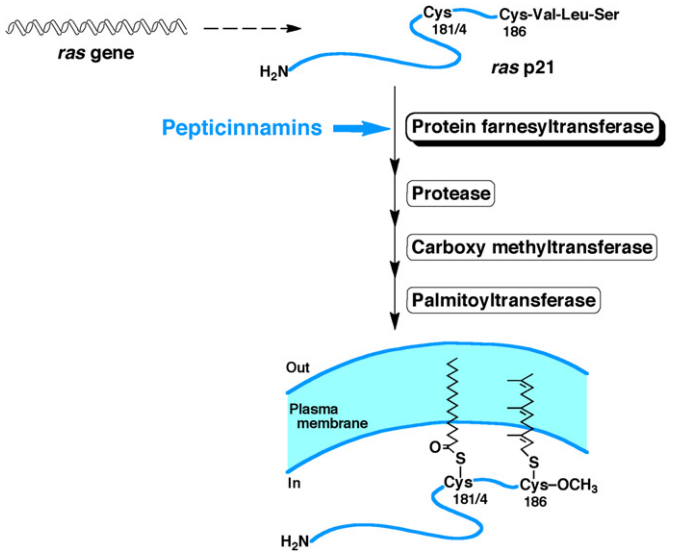


Fig. 8. Post-translational modification of *ras* p21.

Pepticinnamin E (**49**) was found to be an inhibitor of farnesyltransferase isolated from the human monocyte, THP-1.[110,111] The stereochemistry of pepticinnamin E was revealed through the total synthesis of stereoisomers of (**49**) by Dr. H. Waldmann et al.[112]

The fungal metabolites, andrastins A (**50**)~D with *ent* 5α,14β-androstane skeleton, were also discovered using the same screening process.[113–115] The absolute configuration of the *p*-bromobenzoyl derivative of andrastin A was elucidated by X-ray crystallographic analysis.[114]

3.15. Pyripyropenes A (51)~R; ACAT inhibitors

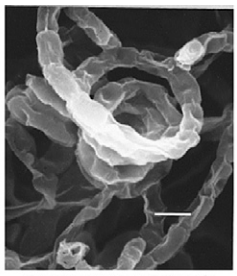
Acyl-CoA; cholesterol acyltransferase (ACAT) is a key enzyme for cholesteryl ester accumulation in atherogenesis, lipoprotein formation in the liver and cholesterol absorption from the intestines. Therefore, ACAT is a promising target for the treatment or prevention of atherosclerosis and hypercholesterolemia. Using an enzyme system for the screening, we discovered the potent ACAT inhibitors, pyripyropenes A (**51**), B (**52**), C (**53**), and D (**54**), isolated from a culture broth of *Aspergillus fumigatus* FO-1289.[116,117] This fungus produces the other 14 related metabolites, namely pyripyropenes E~R.

The relative and absolute stereochemistry of pyripyropene A and E were confirmed by NOE and X-ray crystallographic analysis.[118–120]

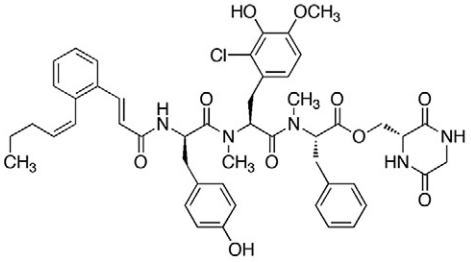
We studied the structure/activity relationship of these compounds intensively and found that the variation of the *O*-acyl group at C-1, C-7, and C-11 caused a dramatic change of ACAT inhibition in cell systems as well as in the enzyme assay.[121–123]

After discovery of pyripyropenes, the two identified forms of acyl-CoA; cholesterol acyltransferase (termed ACAT1 and ACAT2) were characterized.[124] Then, inhibitory activity against these two isozymes was estimated, using CHO cells, and pyripyropene A was found to be specific against ACAT2.[125,126] Pyripyropene A is the first selective inhibitor against the ACAT2 isozyme. It is also of interest that some of the derivatives of pyripyropenes, for example, PP8201 (**55**), show potent insecticidal activity against aphids (unpublished data) and field trials of several derivatives as insecticides are under way.[127]

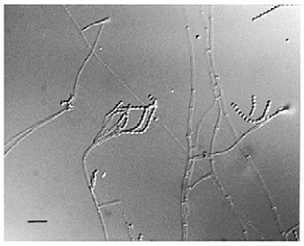
The first total synthesis of pyripyropene A was accomplished through collaboration with Prof. A. B. Smith, III of the University of Pennsylvania, USA[128] (Scheme 2). The total synthesis of the most active member of this family, pyripyropene A, was achieved by a flexible, concise, and highly efficient route via acylation of the known hydroxy α-pyrone (**56**) with α,β-unsaturated acid chloride (**57**), in the presence of an acid catalyst, through isomerization from the *O*-acyl pyrone to the *C*-acyl pyrone and, finally, ring closure with the requisite *anti* geometry at the BC ring fusion of the coupling product (**58**).



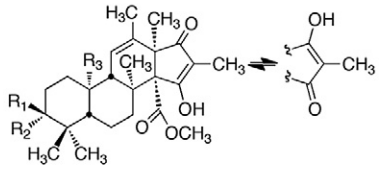
Streptomyces sp. OH-4652



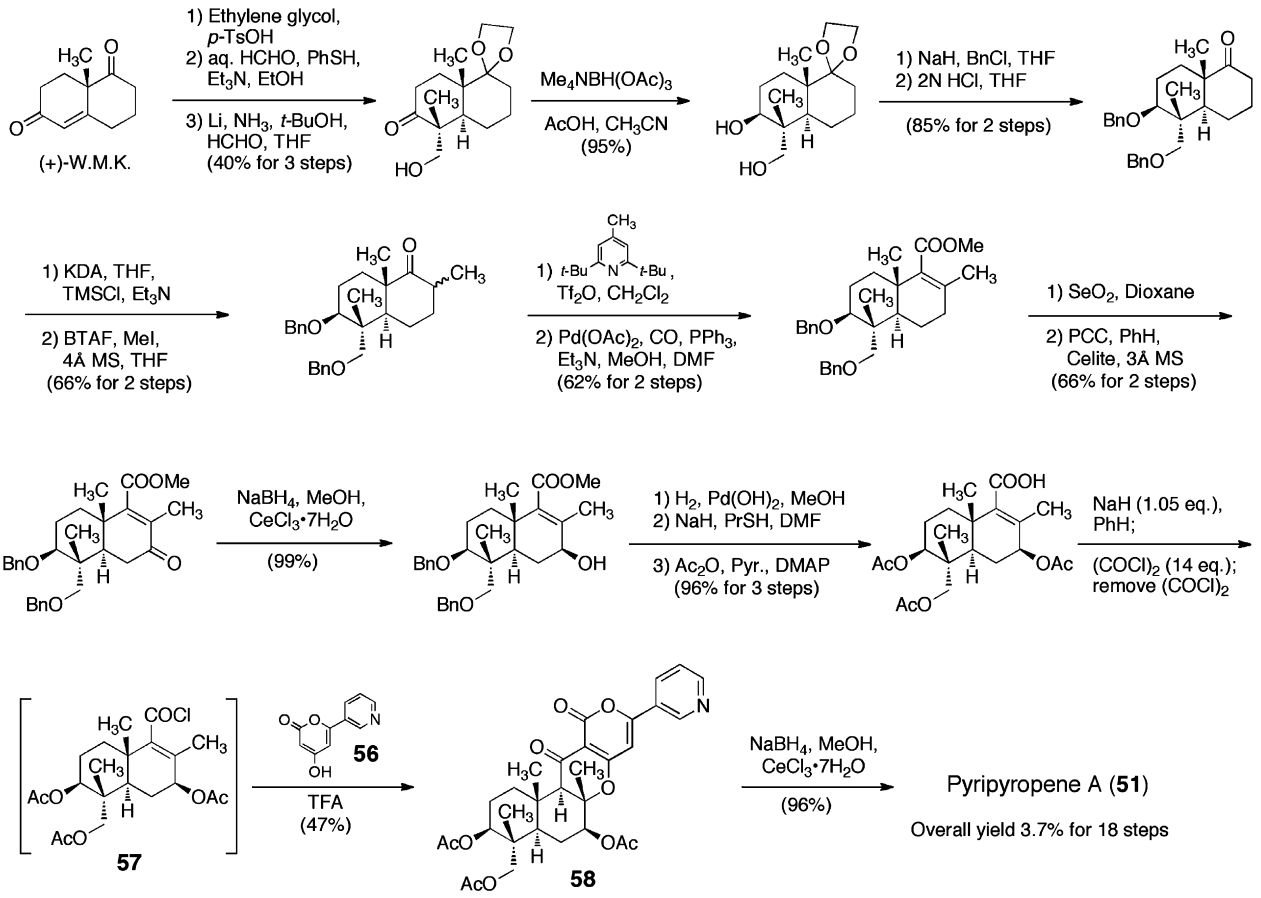
Pepticinnamin E (**49**)



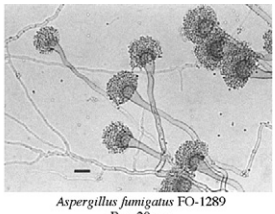
Penicillium sp. FO-3929
Bar: 20 μm



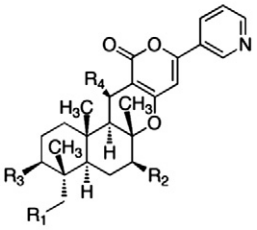
Andrastin A (**50**) (R_1 = OAc; R_2 = H; R_3 = CHO)
B (R_1 = OAc; R_2 = H; R_3 = CH_2OH)
C (R_1 = OAc; R_2 = H; R_3 = CH_3)
D (R_1, R_2 = O; R_3 = CH_3)



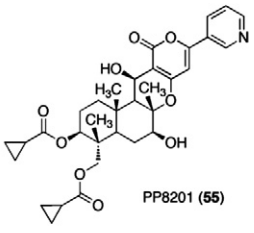
Scheme 2. Total synthesis of pyripyropene A (**51**).



Aspergillus fumigatus FO-1289
Bar: 20 μm



Pyripyropene A (**51**) (R_1 = R_2 = R_3 = OAc; R_4 = OH)
B (**52**) (R_1 = $OCOCH_2CH_3$; R_2 = R_3 = OAc; R_4 = OH)
C (**53**) (R_1 = R_3 = OAc; R_2 = $OCOCH_2CH_3$; R_4 = OH)
D (**54**) (R_1 = R_2 = OAc; R_3 = $OCOCH_2CH_3$; R_4 = OH)
E (R_1 = R_2 = R_4 = H; R_3 = OAc)
F (R_1 = R_2 = R_4 = H; R_3 = $OCOCH_2CH_3$)
G (R_1 = R_2 = H; R_3 = OAc; R_4 = OH)
H (R_1 = R_2 = H; R_3 = $OCOCH_2CH_3$; R_4 = OH)
I (R_1 = R_2 = R_3 = $OCOCH_2CH_3$; R_4 = OH)
J (R_1 = OAc; R_2 = R_3 = $OCOCH_2CH_3$; R_4 = OH)
K (R_1 = R_3 = $OCOCH_2CH_3$; R_2 = OAc; R_4 = OH)
L (R_1 = R_2 = $OCOCH_2CH_3$; R_3 = OAc; R_4 = OH)
M (R_1 = R_3 = OAc; R_2 = $OCOCH_2CH_3$; R_4 = H)
N (R_1 = R_3 = $OCOCH_2CH_3$; R_2 = H; R_4 = OH)
O (R_1 = R_3 = OAc; R_2 = R_4 = H)
P (R_1 = $OCOCH_2CH_3$; R_2 = R_4 = H; R_3 = OAc)
Q (R_1 = R_3 = $OCOCH_2CH_3$; R_2 = H; R_4 = OH)
R (R_1 = OAc; R_2 = R_4 = H; R_3 = $OCOCH_2CH_3$)



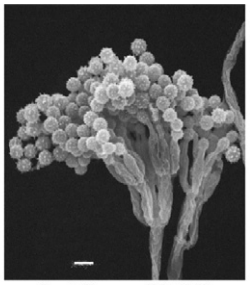
PP8201 (**55**)

3.16. Arisugacins A (59) and B (60); inhibitors of AChE

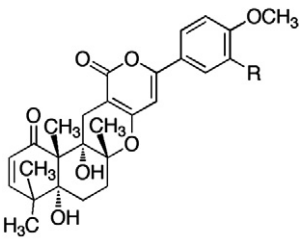
Alzheimer's disease is a degenerative disorder of the central nervous system characterized clinically by the loss of memory, intellect, and cognitive function, a disease that is becoming of increasing significance, especially in aging industrialized societies.

Dr. W. K. Summers et al. reported that an acetylcholine esterase (AChE) inhibitor, tacrine, caused a significant improvement of cognitive function in patients.[129] Soon after their report appeared, we started screening for inhibitors of AChE obtained from human erythrocytes. As a result, the arisugacins A (**59**) and B (**60**), isolated from *Penicillium* sp. FO-5259, were found to be selective AChE inhibitors.[130,131]

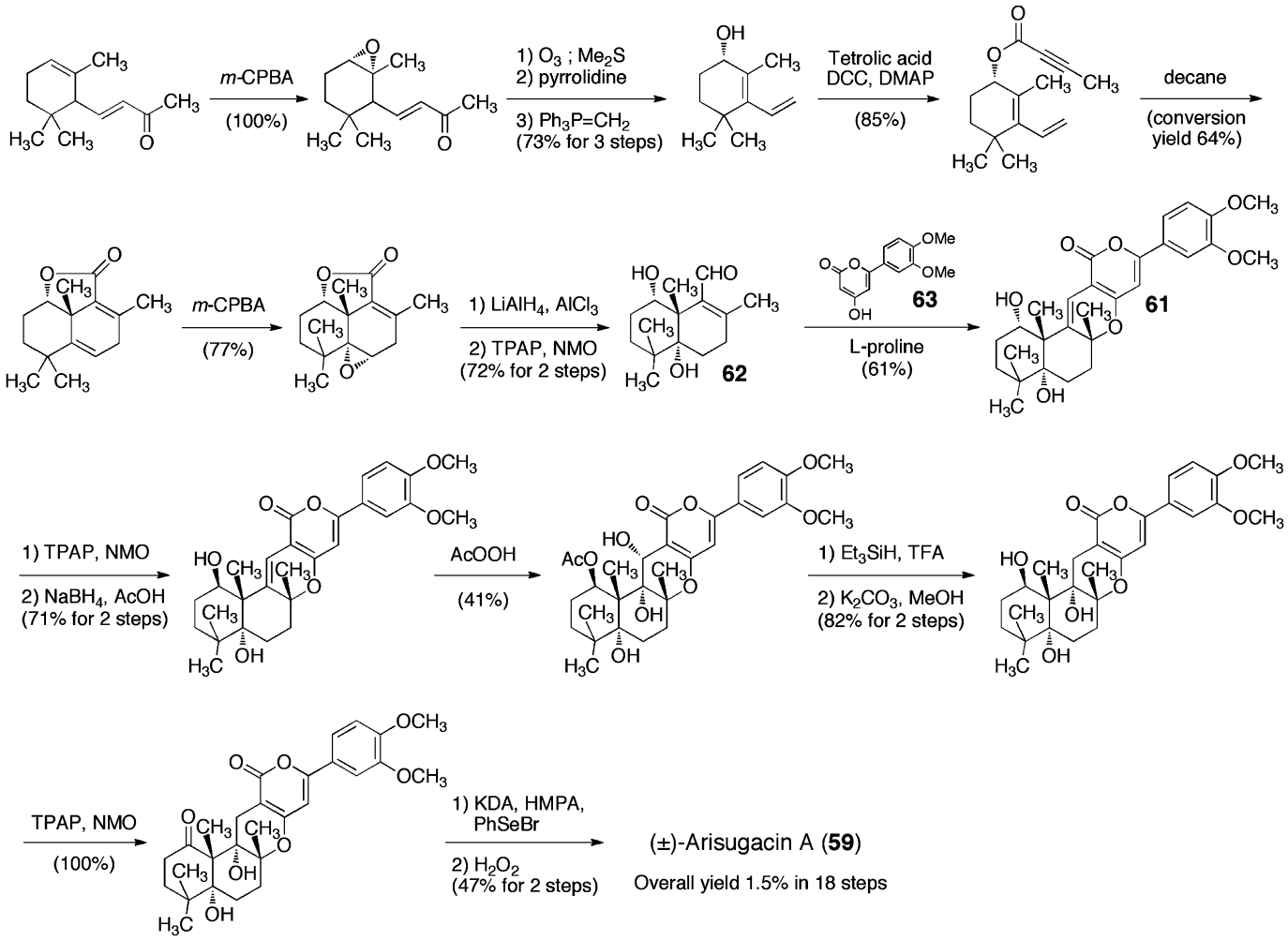
We accomplished the first total synthesis of arisugacins A (**59**) and B (**60**) via a flexible, concise, and highly effective route[132] (Scheme 3). The construction of the advanced olefin (**61**) was envisioned via a Knoevenagel-type reaction of α,β-unsaturated aldehyde (**62**) with the known 4-hydroxy 2-pyrone (**63**) in the presence of L-proline, through amine elimination and 6-electron electrocyclic ring closure.



Penicillium sp. FO-4259



Arisugacin A (**59**) (R = OCH_3)
B (**60**) (R = H)



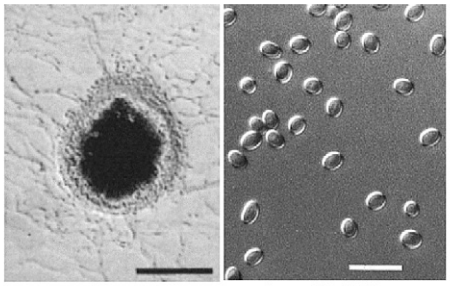
Scheme 3. The total synthesis of arisugacin A (**59**).

3.17. Macrosphelides A (64)~K; cell-adhesion inhibitors

Cell adhesion molecules play important roles in various types of pathological conditions, such as tumor development, allergies, and inflammatory diseases. Therefore, any inhibitor of cell-to-cell adhesion would be an interesting lead compound for development of new agents effective against cancer tumors and/or metastasis, as well as against chronic inflammatory diseases, such as rheumatoid arthritis. Our response to this was to establish a new method of screening for such inhibitors using human leukemia cells (HL-60) and LPS-activated human umbilical vein endothelial cells. As a result, we discovered the macrosphelides (MS) A (**64**)~D, J, and K, produced by *Paraconiothyrium sporulosum* FO-5050, all of which proved to be cell-adhesion inhibitors.[133,134]

Macrosphelides A (**64**) and B (**65**) proved to be the most potent inhibitors. We found that macrospehlide B suppressed lung metastasis of B16/BL6 mouse melanoma cells, doing so by inhibiting cell adhesion to endothelial cells through the sialyl Lewis^X (SLe^x) molecule.[135]

After isolation and structure determination, we quickly managed to synthesize macrospehlide A for further evaluation in vitro and in vivo, as shown in Scheme 4.[136,137] The first asymmetric total synthesis of macrosphelides A and B was accomplished via an asymmetric dihydroxylation of sorbate (**66**), to introduce two chiral centers, and Yamaguchi macrocyclization of seco acid (**67**) to form the 16-membered ring trilactone macrolides. We used single-crystal X-ray analysis and the Mosher NMR method to determine the complete relative and absolute stereochemistry of macrosphelide A, and chemical comparison with artificial macrosphelide B from macrosphelide A to determine the stereostructure of macrosphelide B.

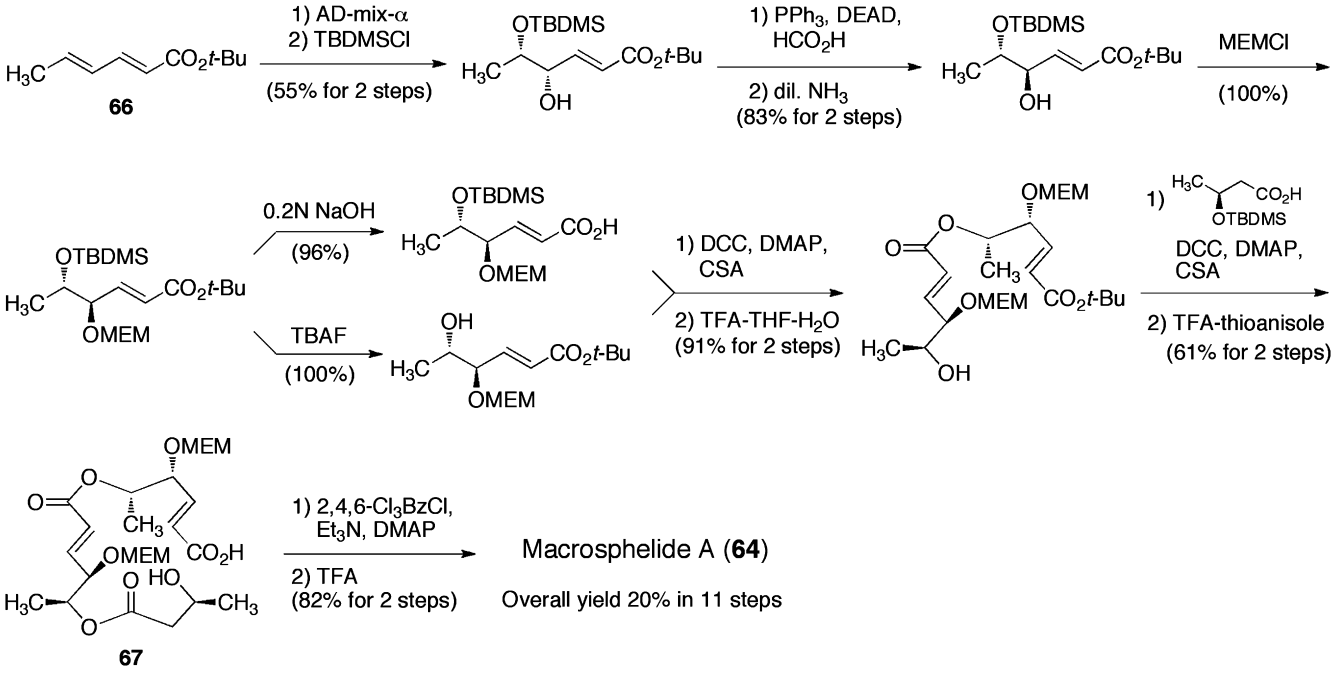


Macrosphelide A (**64**) (R_1 = R_2 = OH; R_3 = H)
B (**65**) (R_1 = OH; R_2, R_3 = O)
C (R_1 = H; R_2 = OH; R_3 = H)
D (R_1 = R_2 = OH; R_3 = H)
(Stereoisomer of A)

Macrosphelide J (R = CH_3)
K (R = CH_2CH_3)

3.18. Madindolines A (68) and B (69); inhibitors of cytokine IL-6

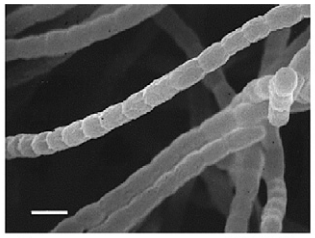
Interleukin-6 (IL-6) is a multifunctional cytokine involved in control of antibody production, T cell activation, hematopoiesis, and acute responses. Disrupted or uncontrolled IL-6 activity causes various serious diseases. It has been reported that excess IL-6 production is closely associated with cancer, cachexia, Castleman's disease, rheumatoid arthritis, hypercalcemia, and multiple



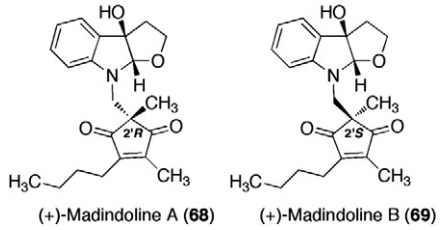
Scheme 4. The asymmetric total synthesis of marosphelide A (**64**).

We have also developed a combinatorial synthesis of macrosphelide, in collaboration with Prof. T. Takahashi of the Tokyo Institute of Technology. Through combinatorial synthesis, a 122-member macrosphelide library has been accumulated, based on a unique strategy for a 3-component coupling, utilizing palladium-catalyzed chemoselective carbonylation and an unprecedented macrolactonization on a polymer support.[138]

myeloma. It therefore seemed sensible to try and find a lead that would facilitate development of a compound that modulates function of this cytokine, preferably via a new action mechanism. We began screening for metabolites that could inhibit the growth of IL-6-dependent cells (MH-60) and found two compounds, madindolines A (**68**) and B (**69**).[139,140] It was subsequently observed that madindoline A binds gp130 and inhibits IL-6 action.[141]



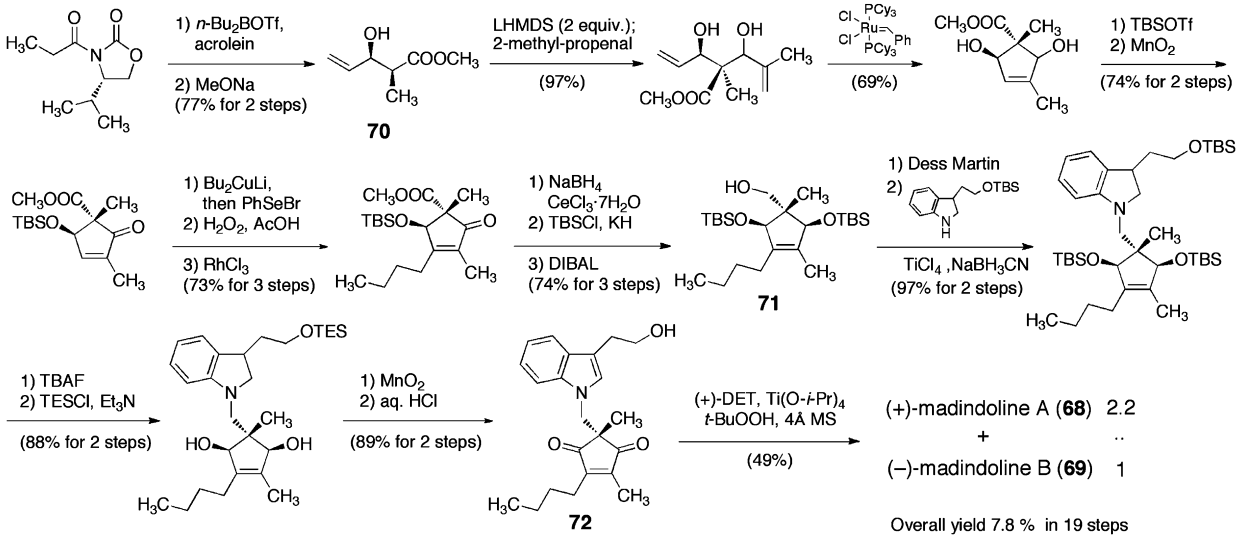
Streptomyces nitrosporeus K930711



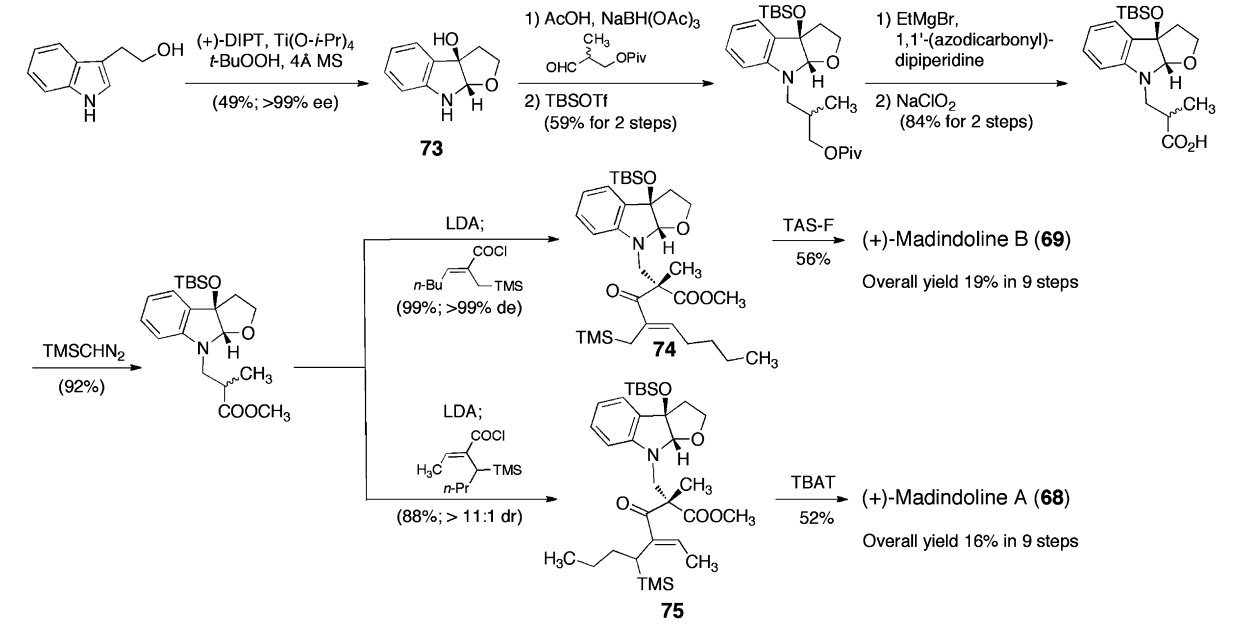
(+)-Madindoline A (**68**) (+)-Madindoline B (**69**)

In order to obtain enough of these compounds for proper biological evaluation, we needed to synthesize madindolines, as naturally occurring amounts of the product were insufficient. We eventually accomplished first- and second-generation total syntheses of both madindolines A and B (Scheme 5 and Scheme 6). In the first-generation synthesis, we completed determination of the absolute stereochemistry of the compounds via an approach using asymmetric oxidative ring closure to construct the chiral 3α-hydroxyfuroindoline moiety of madindolines from indolealcohol.[142] As our point of departure, the Evans asymmetric aldol reaction, followed by methanolysis, afforded β-hydroxyester (**70**) as a chiral building block. The reductive amination between the aldehyde, prepared from the fully substituted cyclopentene moiety (**71**), and the indoline unit, followed by oxidation, afforded the *N*-alkylated indole ethanol (**72**), which was subjected to stereocontrolled oxidative ring closure to furnish the madindolines.

In the second-generation synthesis we achieved a highly convergent short total synthesis of the madindolines by exploiting the 3α-hydroxyfuroindoline unit (**73**), to permit rapid and efficient



Scheme 5. The first total syntheses of madindolines A (**68**) and B (**69**).



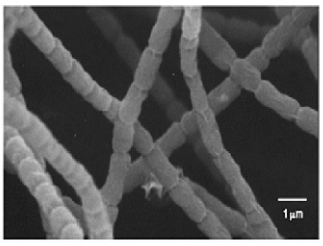
Scheme 6. Second-generation syntheses of madindolines A (**68**) and B (**69**).

construction of the quaternary carbon center of the madindolines, via selective acylation, to afford α,β-unsaturated ketones (**74**) with high diastereoselectivity. Intramolecular cyclization of **74** and **75** permitted construction of the fully substituted diketocyclopentene moiety, creating a process amenable to gram-scale production (Scheme 6).[143]

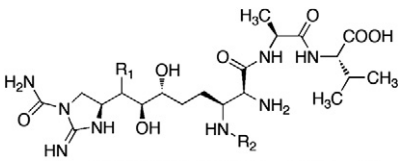
3.19. Guadinomines A (76), B (77), D (80), 28β-OH–factumycin (82), and aurodox (83); inhibitors of type III secretion systems

The type III secretion system (T3SS) is a common virulence system present in many Gram-negative bacteria, including *Yersinia* spp., *Salmonella* spp., *Shigella* spp., *Pseudomonas aeruginosa*, enterpathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and *Chlamydia* spp.[144] These bacteria use the T3SS to deliver effector proteins into the cytosol of the eukaryotic target cell and depend on their respective T3SS to invade the host, resist phagocytosis, grow in deep tissues and thereby cause disease.[145] Recent studies have revealed that the T3SS is not essential for survival of bacteria and is not found in non-pathogenic Gram-negative bacteria, except for some kinds of symbiotic bacteria.

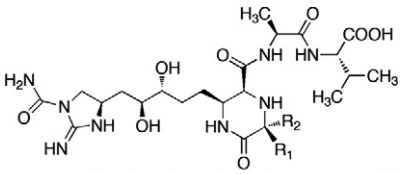
We focused our search for a specific inhibitor of the T3SS, one that is expected to attenuate pathogens specifically and inhibit the process of their infection without killing them or affecting normal human bacterial flora. Such a specific agent would be expected to target only the system involved in the virulence mechanism. Thus, there would be little or no selective pressure for viability, potentially reducing the development of resistance. We employed a convenient assay system in screening for T3SS inhibitors from microbial metabolites, using T3SS-induced hemolysis, developed by Prof. A. Abe of Kitasato University, who is one of our collaborators. Consequently, we discovered several novel compounds, including guadinomines A (**76**), B (**77**), C_1 (**78**), C_2 (**79**) and D (**80**),[146,147] 28α-OH–factumycin (**81**), and 28β-OH–factumycin (**82**).[148] The screen also found a known compound, aurodox (**83**), which potently inhibited T3SS-induced hemolysis (IC50: 1.2 μg/ml). We found that administration of aurodox allowed mice to survive a lethal dose of *Citrobactor rodentium*, a model strain for human pathogens. This in vivo study demonstrated for the first time that a T3SS inhibitor can be applied as a novel class of anti-infective agent.[149]



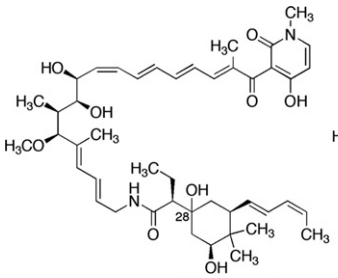
Streptomyces sp. K01-0509



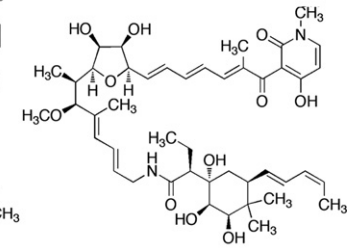
Guadinomine A (**76**) (R_1 = OH; R_2 = H)
B (**77**) (R_1 = R_2 = H)
D (**80**) (R_1 = H: R_2 = Ac)



Guadinomine C_1 (**78**) (R_1 = CH_3; R_2 = H)
C_2 (**79**) (R_1 = H; R_2 = CH_3)



Factumycin [28α-OH (**81**) or β-OH(**82**)]



Aurodox (**83**)

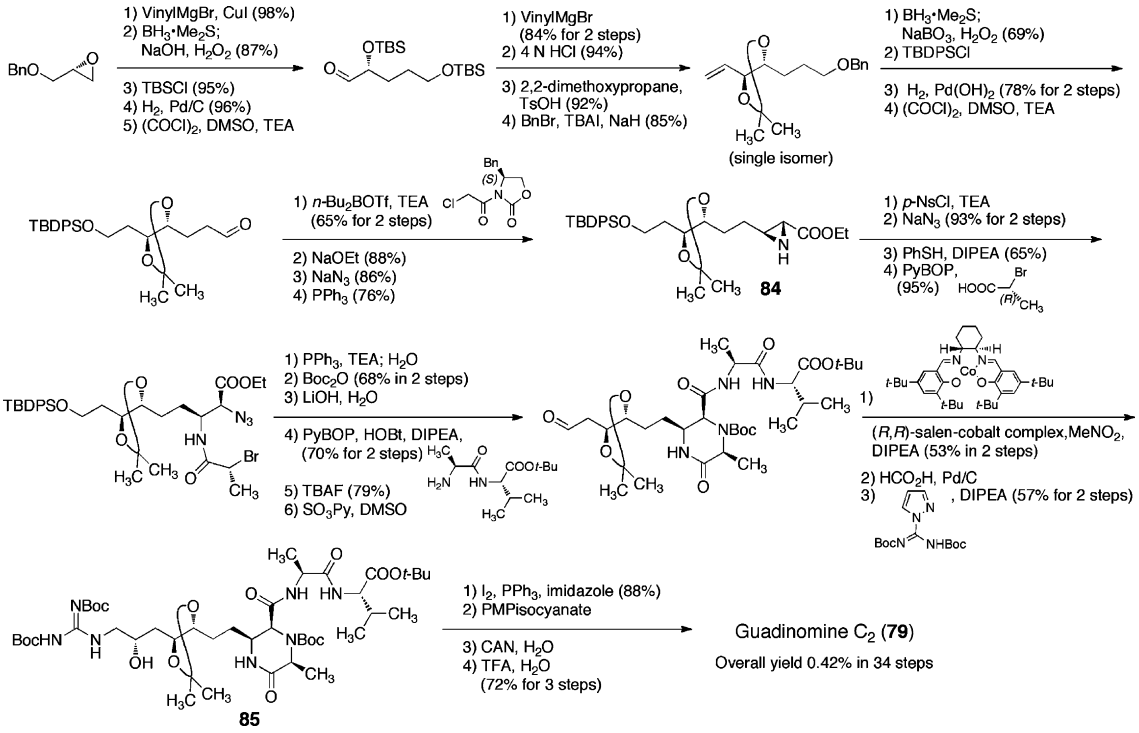
We accomplished the total synthesis of guadinomine C_2 (**79**) (Scheme 7).[150] The first asymmetric total synthesis of guadinomine C_2 was achieved using the novel, concise preparation of tri-substituted piperazinone cores (as optically pure forms) from aziridine (**84**) and S_N2 cyclization to construct the unique five-membered cyclic guanidine substructure from the guanidyl alcohol (**85**). This process not only provides viable routes to the guadinomines, but also establishes the absolute stereochemistry of the natural compound.

3.20. Argifin (86) and argadin (87); cyclic pentapeptide chitinase inhibitors

Chitin occurs in fungi, some algae and many invertebrates, especially insects, but is not found in vertebrates. Thus, chitin synthesis and degradation might be expected to be highly specific primary targets for fungicides and insecticides. We therefore constructed a screening method to identify chitinase inhibitors using a crude preparation of chitinase from *Lucilia cuprina*. In the course of the screening we identified two promising new cyclic pentapeptides, produced by fungi, namely argifin (**86**) and argadin (**87**).[151,152]

Argifin is the first cyclic peptide to demonstrate inhibitory activity against chitinase. It is also the first chitinase inhibitor found of fungal origin. Subsequently, a second cyclic peptide inhibitor, argadin, was identified. Both argifin and argadin showed insecticidal activity against larval stages of *Periplaneta americana* (American cockroach) and *Blattella germanica* (German cockroach). Although both cyclic peptides contain L-arginine, the composition of other amino acids shows major differences. The stereochemistry of argadin for the Cα and Cγ of aspartic β-semialdehyde and Cα of histidine in argadin were elucidated by X-ray crystallography of the argadin-chitinase complex.[153]

The X-ray analysis of both pentapeptide inhibitors and the chitinase complex revealed how the inhibitors' backbone and side chains mimic interaction of the enzyme with the substrate chitooligosaccharides. The structures of argifin and argadin, in complex with *Serratia marcescens* chitinase B, were resolved by X-ray crystallography (2.0-Å resolution) through a collaboration with Prof. D. M. F. van Aalten of the University of Dundee, Scotland.[153,154] These structures provided an unprecedented view of how peptide-based inhibitors inactivate carbohydrate-processing enzymes. For example, the carbonyl oxygen of the histidine in argadin occupies almost the same position as the scissile oxygen in the chitinase β-chitooligosaccharide complex and hydrogen bonds to the catalytic amino acid (Glu144), while Glu144 makes hydrogen bonds to the guanidinium group of the arginine side chain in argifin (Fig. 9).



Scheme 7. The first asymmetric total synthesis of guadinomine C_2 (**79**).

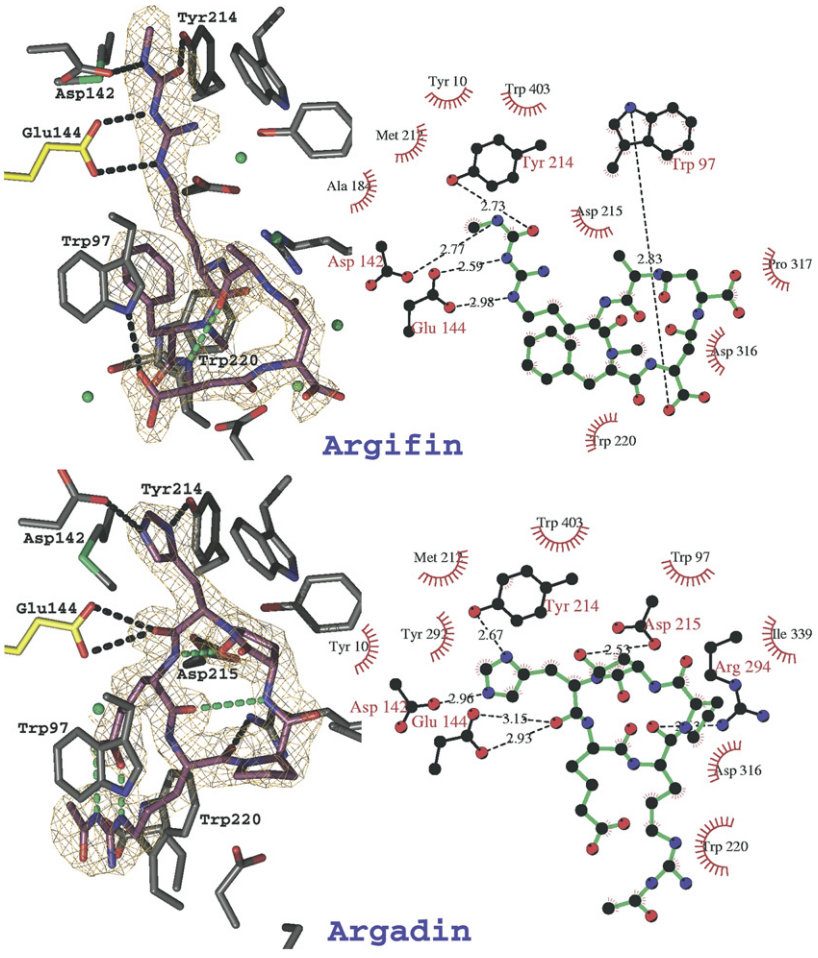
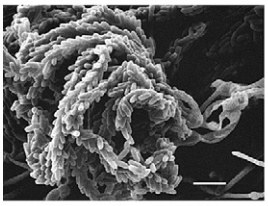
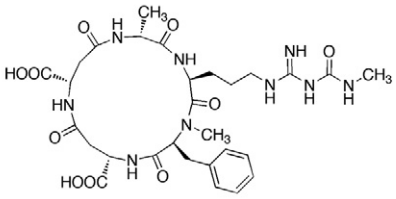


Fig. 9. X-ray crystallography of argifin and argadin in complex with *S. marcescens* chitinase B.

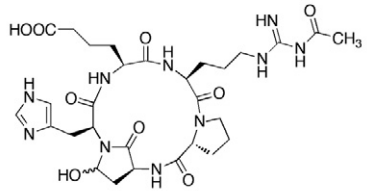
Gliocladium sp. FTD-0668
Bar: 20 μm



Clonostachys sp. FO-7314
Bar: 10 μm



Argifin (**86**)



Argadin (**87**)

From the X-ray crystallography and structure/activity relationship studies, the N^{ω}-methylcarbamoyl-L-arginine moiety of argifin was obviously a crucial component for expression of inhibitory activity against chitinase.

In collaboration with Prof. S. Hirono and Dr. H. Gouda of the School of Pharmacy, Kitasato University, we studied the computer-aided rational molecular design of argifin-derivatives with increased inhibitory activity against chitinase B from *Serratia marcescens* (*Sm*ChiB).[155,156] The molecular dynamic (MD) simulation, the molecular docking calculation and the free-energy analysis of argifins binding in the solution were resolved by the molecular mechanic Poisson-Boltzmann surface area (MM-PBSA) method. The custom-designed argifin-derivatives were synthesized by solid phase synthesis, developed in our laboratory, and their inhibitory activities against *Sm*ChiB were measured. Finally, we were able to obtain a derivative (**88**), which exhibited 28-fold more potency than argifin. In the derivative, the D-Ala of argifin was replaced with D-Leu and the 4-benzylpiperazine was attached to L-Asp.[157]

In order to obtain even more potent derivatives, we tried an in situ Click Chemistry approach using an azide derivative of N^{ω}-methylcarbamoyl-L-arginine. In situ Click Chemistry is a target-guided synthesis technique, developed by Prof. K. B. Sharpless of the Scripps Research Institute in California, USA, for discovering potent protein ligands by assembling azides and alkynes into triazole in situ in the affinity site (a catalytic pocket) of a target protein.[158]

Working in collaboration with Prof. Sharpless, this process revealed a potent and novel 1,5-distributed *syn*-triazole compound (**90**), obtained from an azide bearing arginine derivative (**89**) of argifin. The compound (**90**) (see Fig. 10) expressed 300-fold more potent inhibitory activity against *Sm*ChiB compared to argifin.[159]

3.21. Nafuredin (91) and atpenin A5 (93); inhibitors of energy metabolism

Differences in energy metabolism between the host and helminth parasites were believed to be attractive targets for possible treatment of helminthiasis. The NADH/fumarate reductase system,

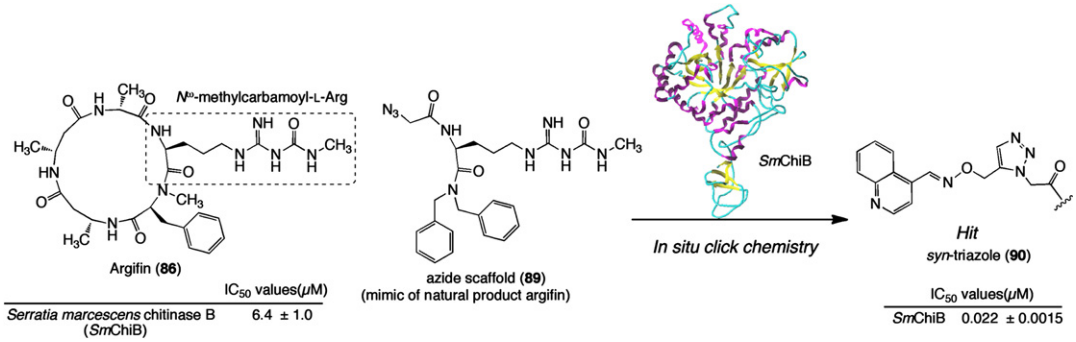


Fig. 10. In situ Click Chemistry to prepare a novel 1,5-distributed *syn*-triazole compound (**90**).

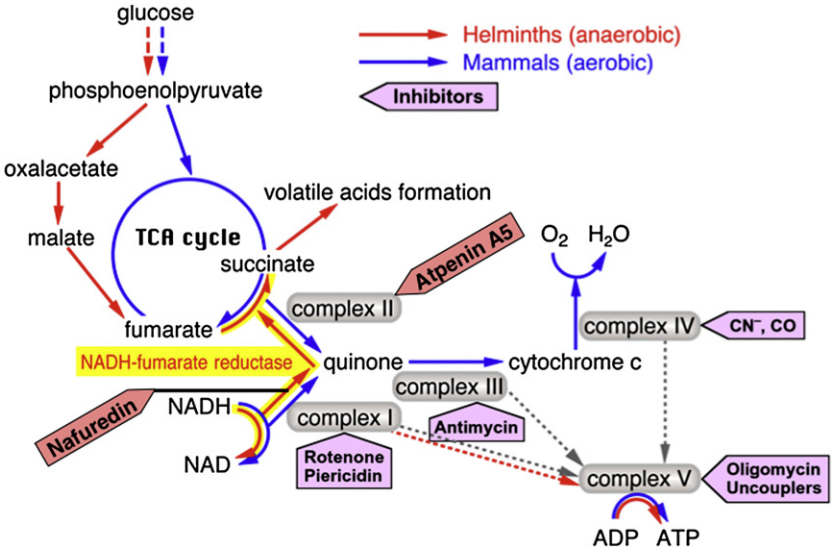
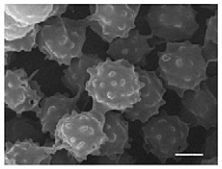
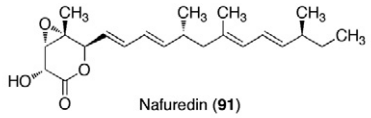


Fig. 11. Energy metabolism and inhibitors.

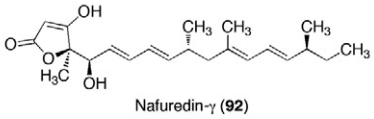
which is found in many anaerobic organisms, is part of a special respiratory system in parasitic helminthes. The system is composed of complex I (NADH/rhodoquinone oxidoreductase) and complex II (rhodoquinol/fumarate reductase) (Fig. 11). Electrons from NADH are accepted by rhodoquinone through complex I, and then transferred to fumarate through complex II. This anaerobic electron transport system can provide ATP in the absence of oxygen. We screened for inhibitors of NADH/fumarate reductase using *Ascaris suum* (roundworm) mitochondria and obtained a novel antibiotic, nafuredin (**91**), produced by an *Aspergillus niger* FT-0054 strain isolated from a marine sponge.[160,161]



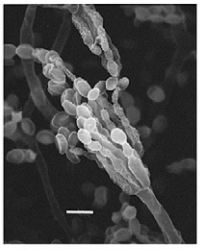
Aspergillus niger FT-0554
Conidia Bar: 2 μm



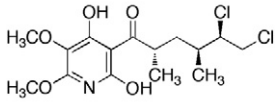
Nafuredin (**91**)



Nafuredin-γ (**92**)



Penicillium sp. FO-125
Bar: 5 μm



Atpenin A5 (**93**)

Through collaborative study with Prof. K. Kita, the University of Tokyo, nafuredin was revealed to inhibit NADH/fumarate reductase and NADH/rhodoquinone oxidoreductase of *A. suum* at nanomolar concentrations, while it showed only weak inhibition of rhodoquinol/fumarate reductase (Table 1). Therefore, nafuredin is a complex I inhibitor, although it inhibits rat liver complex I (NADH/ubiquinone oxidoreductase) only at very high concentration. Interestingly, nafuredin inhibits not only anaerobic adult complex I of *A. suum*, but also larval complex I, which has an aerobic energy metabolism like that of mammals. Thus, nafuredin is a selective inhibitor of helminth complex I.

Nafuredin shows anthelminic activity against *Haemonchus contortus* (Barberpole worm) in in vivo trials using sheep. This anthelmintic activity of nafuredin may thus be due to the inhibition of complex I (Table 1).

Nafuredin is a promising novel anthelmintic lead compound and we have accomplished its total synthesis.[162] Recently, we found that nafuredin is converted to a novel γ-lactone derivative (named nafuredin-γ (**92**)) under mild basic conditions, and this derivative has the same enzyme inhibitory and anthelmintic activity as the parent compound. Since the synthesis of the lactone moiety of nafuredin-γ is simpler than that of nafuredin, having the five-membered ring lactone nafuredin-γ is useful for use as a lead compound for development of a new, potent anthelmintic. We have also achieved the total synthesis of nafuredin-γ.[163]

Using the same screening system allowed us to 're-discover' atpenin A5 (**93**) and related compounds as NADH/fumarate reductase inhibitors. Atpenins were originally isolated by our group from the culture broth of *Penicillium* sp. FO-125 as lipid metabolism effectors.[164] In contrast to nafuredin, atpenins inhibit complex II (succinate/ubiquinone oxidoreductase), and the inhibition is non-selective between helminthes and mammals.[165] Although there are strong inhibitors of complexes I, III, and IV, no very potent inhibitors of complex II had previously been described. The IC_{50} value of atpenin A5 is 300-fold lower than that for carboxin, the most potent known complex II inhibitor.

Mitochondrial complex II is an integral membrane protein, comprising four subunits (Fig. 12). The largest subunit (Fp) is the FAD flavoprotein of some 70 kDa. The dehydrogenase catalytic

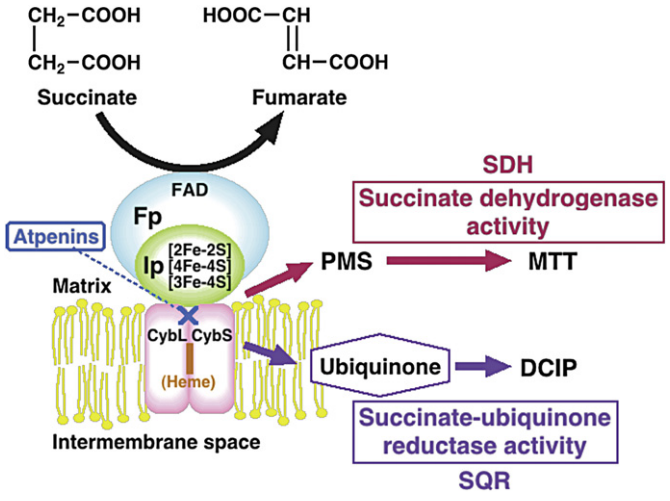


Fig. 12. Subunit structure and enzyme activities of complex II.

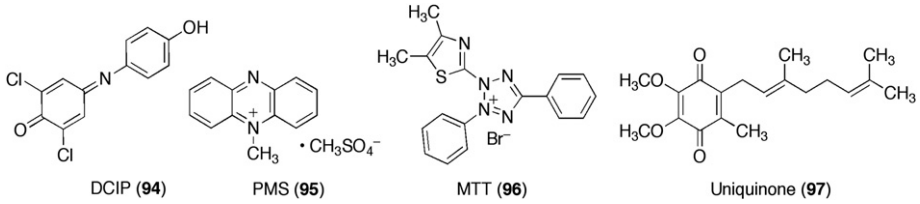
Table 1
Effect of nafuredin on electron transport enzymes of *A. suum*, *H. contortus*, and rat liver

	IC_{50} (nM)					
	Complex	*A. suum* (adult)	*A. suum* (L2)	*H. contortus* (adult)	*H. contortus* (L3)	Rat liver
NADH/fumarate reductase	I+II	12	[a]	[a]	[a]	1,000
NADH/ubiquinone reductase	I	8	8.9	86	120	10,000
NADH/rhodoquinone reductase	I	24	9.0	195	350	>100,000
Rhodoquinol/fumarate reductase	II	80,000	[a]	[a]	[a]	[a]
Succinate/ubiquinone reductase	II	>100,000	[a]	[a]	[a]	>100,000

[a] Not tested.

portion consists of Fp and another subunit (Ip) of approximately 30 kDa, which contains three different types of iron-sulfur complex. The small hydrophobic subunits (CybL and Cybs) anchor the catalytic portion to the mitochondrial inner membrane and are also involved in electron transfer to quinones.

The complex II catalyzing reaction can be separated into two distinct components, succinate/ubiquinone reductase activity (SQR) and succinate dehydrogenase activity (SDH) (Fig. 12). SQR, which is a physiological reaction, can be estimated with 2,6-dichlorophenolindophenol (DCIP) (**94**), a redox indicator. The SDH reaction is a partial reaction of complex II and is catalyzed by the Fp and Ip subunits. It occurs in non-physiological conditions and can be estimated with the electron acceptor phenazine methosulfate (PMS) (**95**) and another redox indicator, 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2*H*-tetrazolium bromide (MTT) (**96**). We found that atpenin A5 inhibits potently and specifically both the SQR and SDH components of mitochondrial complex II.[165] From this evidence, we could predict that atpenin A5 binds to the central position of complex II, that is, the binding site of ubiquinone (**97**).

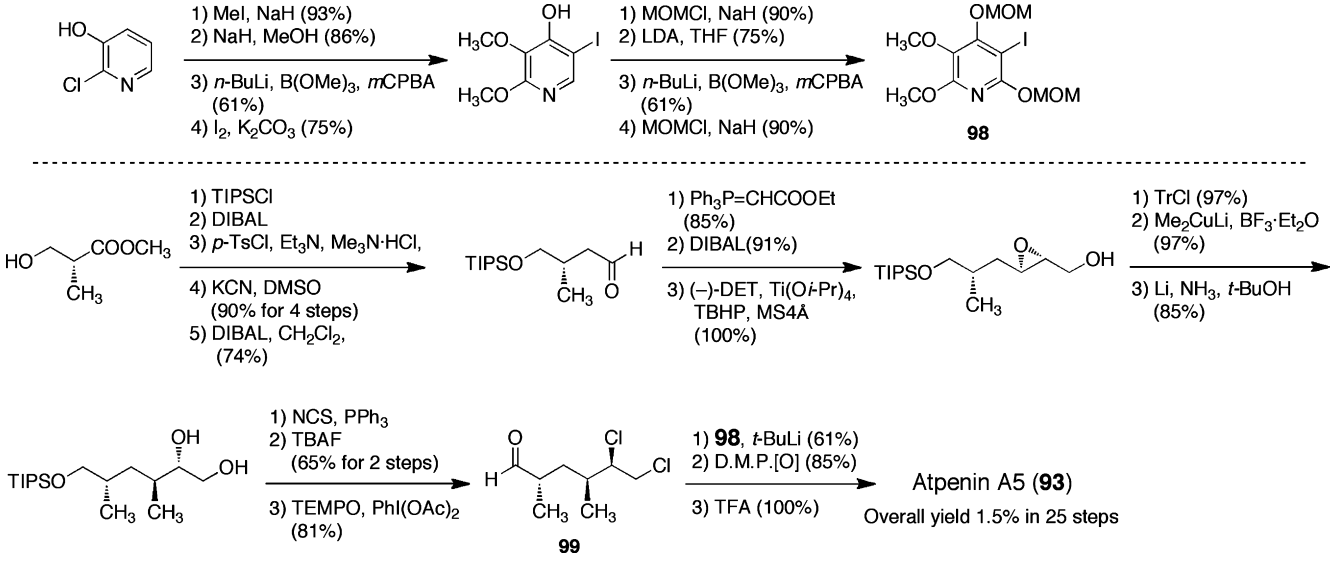


We were able to clearly resolve the binding mechanism of atpenin A5 (**93**) to *E. coli* SQR by X-ray crystallography, in collaboration with Prof. S. Iwata of Imperial College, London.[166] Atpenin A5 was, as expected, located within the same hydrophobic pocket as ubiquinone, but at a different position within the pocket of *E. coli* SQR. Atpenin A5 was bound deeper into the site, prompting further assessment using protein–ligand docking experiments *in silico*.

The total synthesis of atpenin A5 was accomplished by Prof. T. Nagamitsu, a member of our collaborative research group at Kitasato (Scheme 8).[167] Enantioselective total synthesis of atpenin A5 was achieved using a convergent approach involving a coupling reaction between 5-iodo-2,3,4,6-tetraalkoxypyridine (**98**) and a side chain aldehyde (**99**). The two key segments were synthesized through *ortho*-metalation/boronation with $(MeO)_3B$/oxidation with *m*CPBA, *ortho*-iodination, the halogen dance reaction, Sharpless epoxidation and regioselective epoxide-opening reactions. This synthetic study resulted in a revision of the earlier reported ^1H NMR data of the natural atpenin A5 and confirmation of the stereochemistry of the molecule.

3.22. Actinohivin (100); an inhibitor of HIV entry to cells

The development of a topical microbicide capable of blocking the sexual transmission of HIV is urgently needed to help control the global HIV/AIDS pandemic. We have searched for anti-HIV substances using a syncytium formation assay system, constructed with HeLa/CD4/Lac-Z cells (expressing the cellular receptors CD4 and CXCR-4) and HeLa/T-env/Tat cells (expressing the viral envelope glycoprotein complex gp120/gp41). Incubating a combination of these two cell types, we subsequently discovered a novel anti-HIV lectin that inhibits syncytium formation, which we designated as actinohivin (**100**). It was isolated from a culture broth of a soil microbe, actinomycete strain K97-0003.[168–171] Later, we found this strain to be a new genus and named it *Longispora albida* K97-0003.[170] Actinohivin exhibits potent anti-HIV activity in various strains of T-tropic and M-tropic HIV-1 and HIV-2 (IC_{50}=2–110 nM), inhibiting viral entry to cells by binding to the high-mannose type glycans (HMTGs) of gp120.[172,173] Actinohivin has a unique sequence consisting of 114 amino acids and a highly conserved internal sequence triplication (comprising amino acids 1–38, 39–77, and 78–114; segments 1–3, respectively). These three segments are necessary for potent anti-HIV activity. We were able to clone the gene encoding actinohivin and established a production system of recombinant actinohivin in *E. coli*.[170]



Scheme 8. Total synthesis of atpenin A5 (**93**).

X-ray crystallographic analysis of actinohivin revealed a 3D structure containing three sugar-binding pockets (Fig. 13). The strong specific affinity of actinohivin to gp120 is considered to be due to multivalent interaction of the three sugar-binding pockets with three HMTGs of gp120 via the 'cluster effect' of lectin.[173] Actinohivin is a good candidate for investigation and holds promise for development of a safe microbicide to help prevent HIV transmission.

The second compound identified by this screening system was calpinactam (**103**) produced by *Mortierella alpina* FKI-4905.[176] Calpinactam is a unique hexapeptide with a caprolactam ring at the C-terminal. Calpinactam selectively inhibited the growth of various mycobacteria, displaying MIC values against *M. smegmatis* and *M. tuberculosis* of 0.78 and 12.5 μg/mL, respectively.

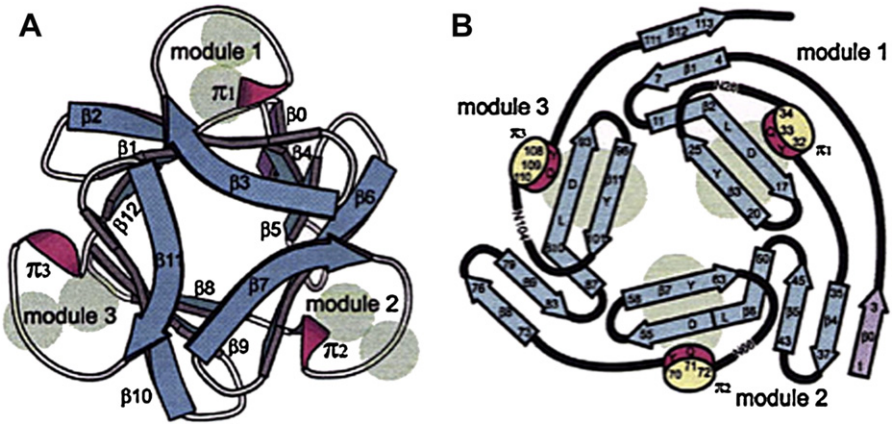
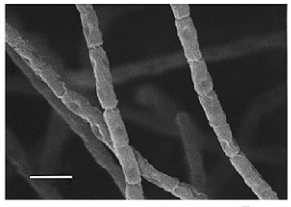


Fig. 13. Tertiary structure (A) and secondary structure topology (B) of actinohivin (**100**). Ellipsoids colored light gray in B indicate the three mannose-binding pockets.



Longispora albida K97-0003^T

```
1- 38  ASVTIRNAQTGRLLDSNYNGNVYTLPANGGNYQRWTGP
39- 76  GDGTVRNAQTGRCLDSNYDGAVYTLPCNGGSYQKWLFY
77-114  SNGYIQNVETGRVLDSNYNGNVYTLPANGGNYQKWYTG
```

Actinohivin (**100**)

3.23. Lariatins A (101) and B (102), calpinactam (103); selective inhibitors of the growth of mycobacteria

Tuberculosis remains one of the world's most serious infectious diseases, in tropical developing countries as well as in the industrialized nations. Unsurprisingly, we have been searching for antituberculosis agents from the outset. In our screens, direct use of *Mycobacterium tuberculosis* is not particularly suitable because of its pathogenicity. Therefore, we opted to concentrate on use of the closely related, non-pathogenic *Mycobacterium smegmatis*, as an alternative test organism. We discovered that lariatin, produced by *Rhodococcus jostii* K01-B0171, is a selective inhibitor of *M. smegmatis*.[174]

Lariatins A (**101**) and B (**102**) are unique cyclic peptides, consisting of 18 and 20 L-amino acid residues, respectively, with an internal linkage between the γ-carboxyl group of Glu8 and the α-amino group of Gly1. The 3-dimensional structure of lariatin A, deduced from NMR data, indicates that the tail segment (Trp9-Pro18) passes through the ring segment (Gly1-Glu8) to form a 'lasso' structure (Fig. 14).[175] Lariatin A also inhibits the growth of *M. tuberuclosis* with an MIC of 0.39 μg/ml in the liquid microdilution method.

3.24. Cyslabdan (104); a potentiator of imipenem activity

Cyslabdan (**104**) was originally identified through our Chemical Screening operations. We later found it to be a potentiator of imipenem activity against multidrug-resistant *Staphylococcus aureus*

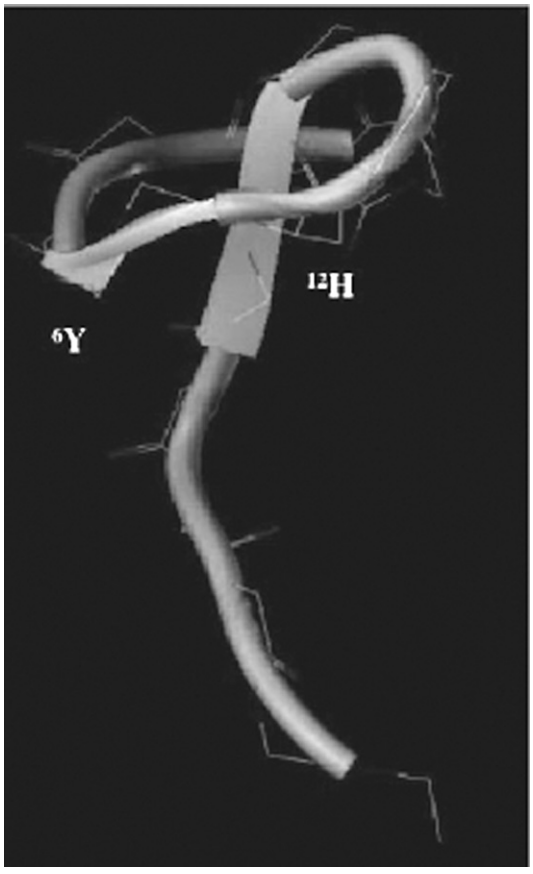
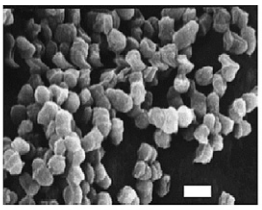
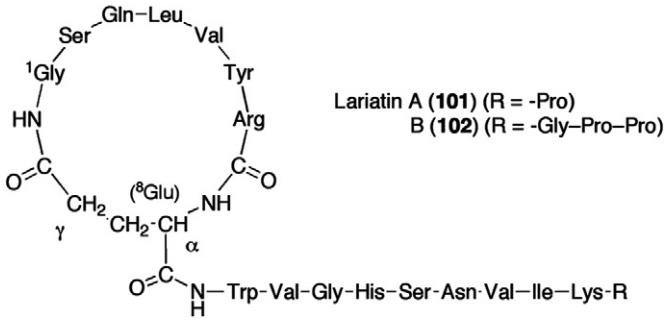


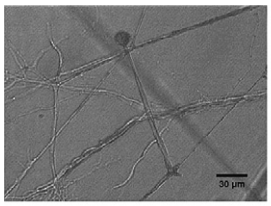
Fig. 14. Lasso structure of lariatin A (**101**).



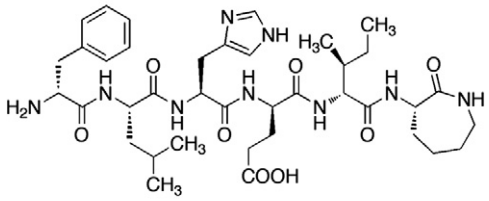
Rhodococcus jostii K01-B0171



Lariatin A (**101**) (R = -Pro)
B (**102**) (R = -Gly–Pro–Pro)



Mortierella alpina FKI-4905

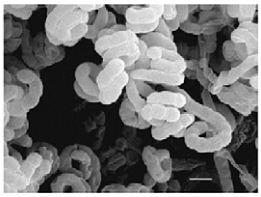


Calpinactam (**103**)

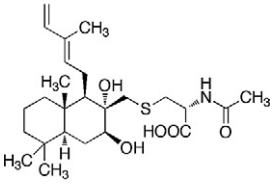
(MRSA).[177] Cyslabdan has a labdane-type diterpene skeleton connecting with an *N*-acetylcysteine via thioether. The relative configuration of the diterpene part was determined by NOE experiments and the absolute configuration of *N*-acetylcysteine part was elucidated as the L-form by HPLC analysis using a chiral column.[178]

3.25. Verticilide (105); a ryanodine-receptor binding inhibitor

Among our various screening systems, we have systems targeting receptor binding of a specific ligand. One of them is for identifying ryanodine-receptor binding inhibitors. Ryanodine (**106**)

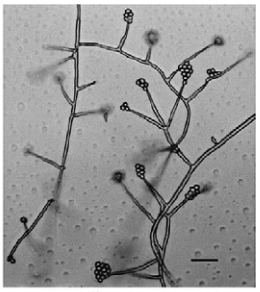


Streptomyces sp. K04-0144

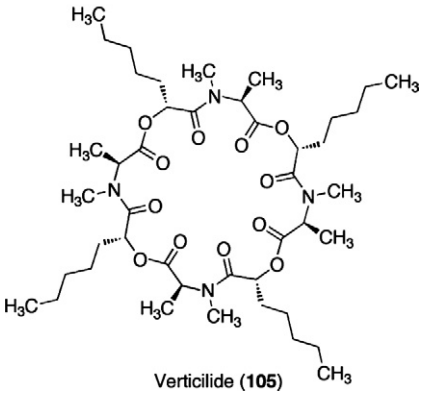


Cyslabdan (**104**)

was isolated from a plant, *Ryania speciosa*, and exhibits insecticidal activity, and its receptor had been found. Therefore, we screened for antagonists or agonists from microbial metabolites using the ryanodine receptor from the cockroach and discovered verticilide (**105**), a depsipeptide possessing antagonistic activity and a peculiar structure not previously observed.[179]



Verticillium sp. FKI-1033
Bar: 10 μm



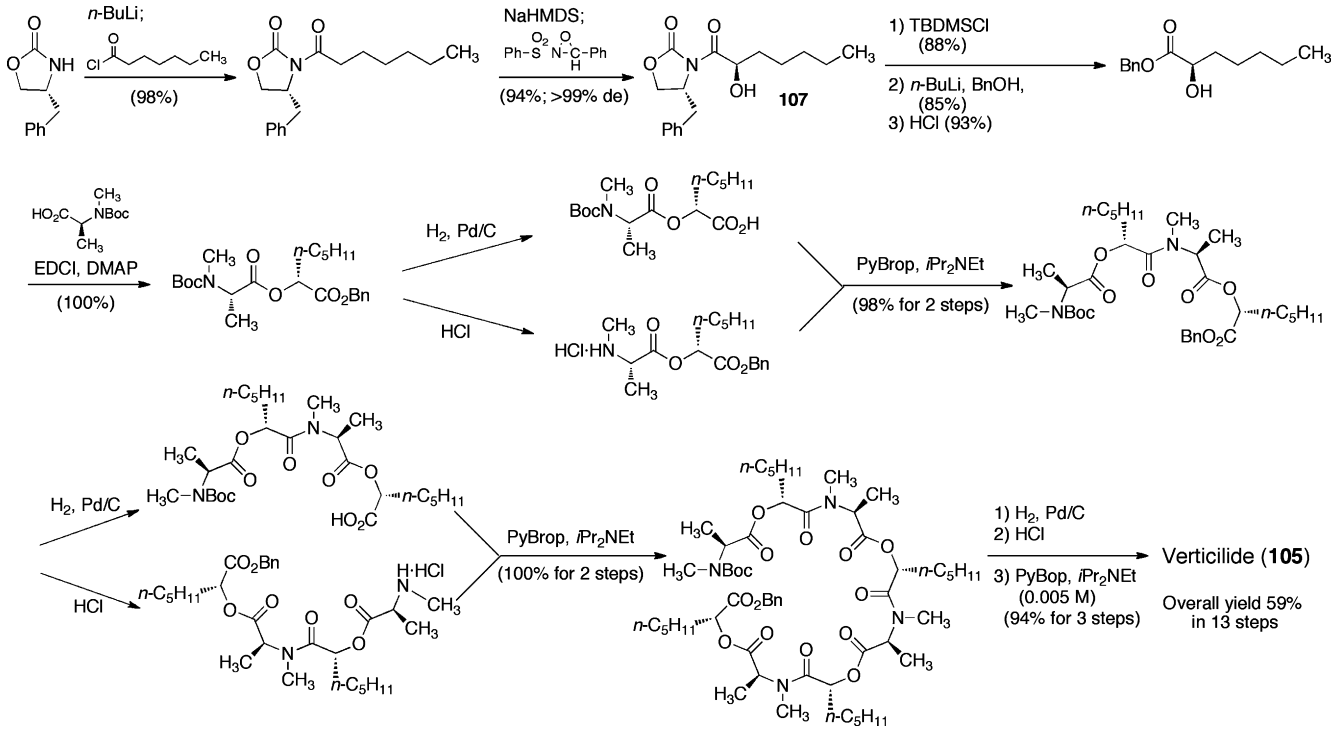
Verticilide (**105**)

Our group achieved the total synthesis of verticilide and we obtained sufficient quantities to allow us to evaluate it as an insectide.[180] The first total synthesis and elucidation of the stereochemistry of verticilide was accomplished via a diastereoselective Davis oxidation to prepare the optically pure 2-hydroxyheptanate (**107**). We have synthesized 10 g of verticilide using this technique, thereby allowing further testing to proceed (Scheme 9).

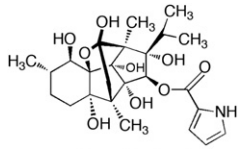
4. The biosynthesis of microbial metabolites

4.1. Studies on precursor incorporation into microbial metabolites

As a result of the variety of new screening systems we have devised and introduced since the early-1970s, we have managed to



Scheme 9. The total synthesis of verticilide (**105**).



Ryanodine (**106**)

discover a significant number of new compounds, many possessing novel structures and skeletons—a few beyond what we could have possibly imagined. We have also managed to make great headway toward a better understanding of how microorganisms biosynthesize the compounds, what precursors they use and by what methods they accomplish the production.

Initially, we examined what precursors were being used in the biosyntheses of microbial metabolites. At the time, research was transitioning between the use of precursors with radioactive isotopes toward using small molecular weight compounds with particular elements labeled with a stable isotope, such as ^{13}C. In those early days, as biosynthetic precursors containing stable isotopes commercially available were very limited, we synthesized our own labeled compounds using $^{13}CO_2$, etc., as raw materials.

In the early-1970s, ^{13}C NMR spectrometry was introduced and used in chemical analysis of natural products with complex structure. For us, this was extremely fortuitous and timely and we were able to exploit the technique in our biosynthetic studies for clarification of how and where ^{13}C-precursors were incorporated into the molecular structure of microbial products. By collaborating with Prof. G. Lukacs of The Institut de Chimie des Substances Naturelles du CNRS, France, we initiated biosynthetic studies with ^{13}C NMR spectrometry on several macrolide antibiotics, as well as carrying out their structure determination.[181–184]

At first, we examined the skeleton formation of the lactone moiety of 16-membered ring macrolides. By addition of ^{13}C-labeled precursors into the culture medium for the fermentation of antibiotic-producing microorganisms we could deduce the means by which the precursors were incorporated into the lactone moiety. In this way, it was clarified that the carboxyl carbon of *n*-butylate is incorporated into both the C-6 positions of leucomycin and tylosin and that their aldehyde residues are derived from the methyl residue of *n*-butylate (Fig. 15). This was the first evidence that the C4-unit is incorporated into the polyketide skeleton. Afterwards, we also clarified that the intact C6-unit is used for formation of the polyketide skeleton in phthoramycin (Fig. 16).[185]

Xanthoquinodin (**108**) is the first heterodimer in which octaketide-derived xanthone and anthraquinone monomers are connected in an 'end-to-body' fashion. In view of the biosynthesis of xanthoquinodin (Fig. 17),[186] an agent having anticoccidial activity, it is clear that producing organisms can change freely both the skeleton constructed by incorporation of the precursors as well as the synthesis of any specific final product.[187]

The studies of ^{13}C-precursors and analysis of biosynthetic genes made it clear that microorganisms form skeletons with complicated structures through synthesis involving a variety of precursors derived from amino acids and so on.

Some selected instances below illustrate how some unique skeletons are formed by diverse precursors. These are examples which have served to guide the production of non-natural products through genetic manipulation and other methods.

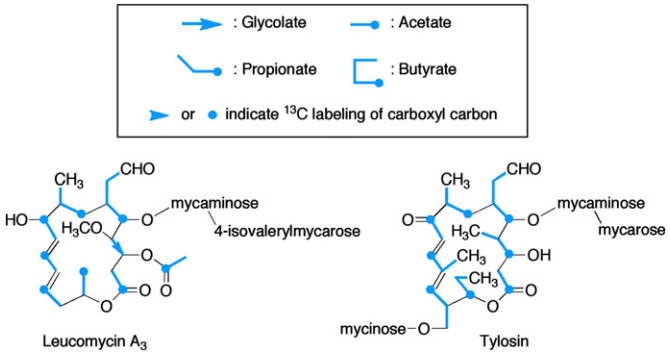


Fig. 15. Incorporation of a ^{13}C-precursor into leucomycin A_3 (**1**) and tylosin (**4**).

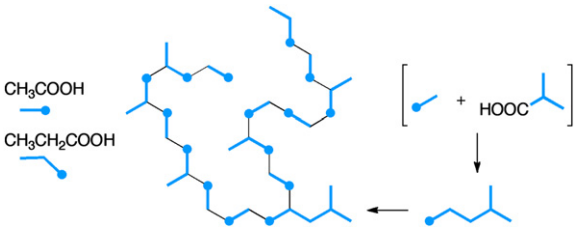


Fig. 16. Proposed biosynthesis of phthoramycin (**36**).

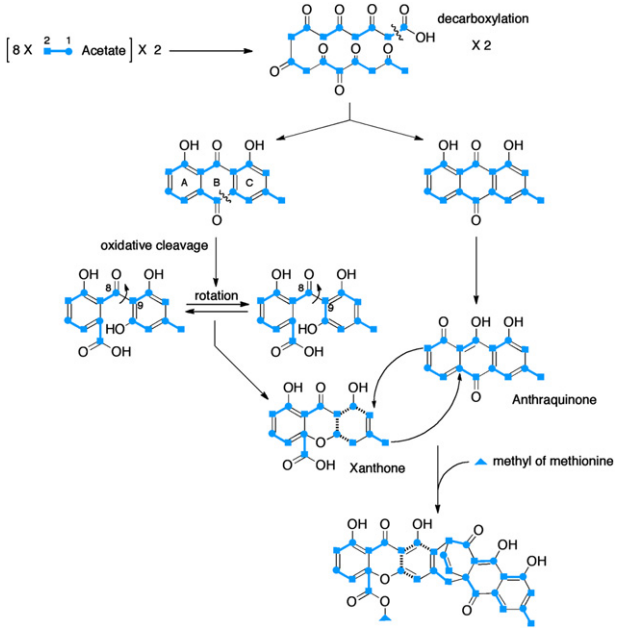
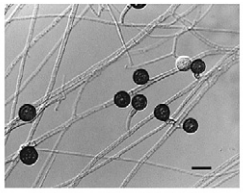
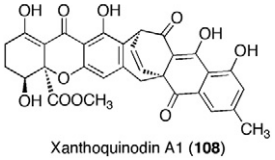


Fig. 17. Biosynthesis of the anticoccidial antibiotic xanthoquinodin (**108**).



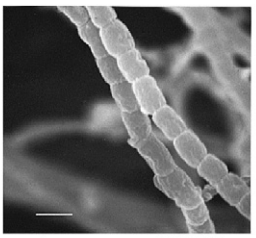
Humicola sp. FO-888
Bar: 20 μm



Xanthoquinodin A1 (**108**)

The cytocidal antibiotics furaquinocins A (**109**) and B (**110**) are derived from a pentaketide, two mevalonates and two C1-units of L-methionine (Fig. 18).[188]

Aurantinins A (**111**) and B (**112**) were found to originate from *Bacillus aurantinus* during the course of screening of antibiotics produced by eubacteria, and have unique structures with side chains on four rings.[189–191] As shown in Fig. 19, instead of the propionate pathway (which does not function), the two C1-units attached to the carbon come from the acetate carboxyl in the polyketide chain, derived from the methyl group of an acetate, and the five C1-units located at the tail of the acetate unit are derived from methionine.[192,193] This biosynthetic mechanism exhibits an interesting difference as, in actinomycetes, the methyl residue on a polyketide chain is usually derived from the propionate unit.



Streptomyces sp. KO-3988

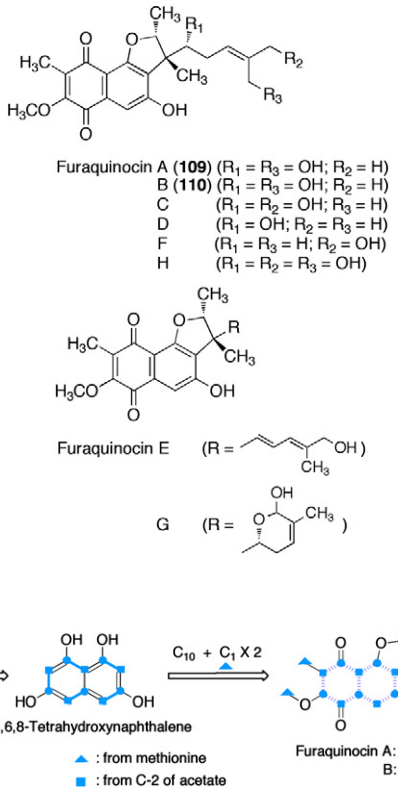
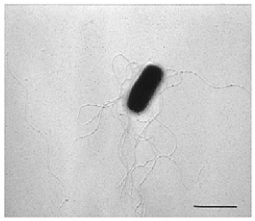
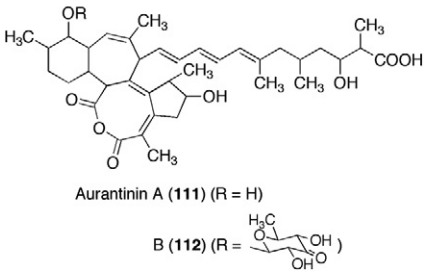


Fig. 18. Biosynthesis of furaquinocins A (**109**) and B (**110**).

An inhibitor of ACAT2, pyripyropene A (**51**) has a unique structure containing a pyridine moiety. Incorporation of ^{13}C-labeled precursors and degradation experiments on pyripyropene A found that a nicotinic acid primer condenses with two acetates in a head-to-tail fashion, forming the pyridino-α-pyrone moiety, which is linked



Bacillus aurantinus KM-214



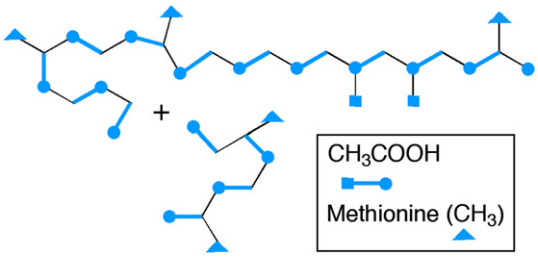


Fig. 19. Biosynthesis of aurantinin A (**111**).

with a sesquiterpene to create the core skeleton. Then, three acetyl residues are introduced into the core (Fig. 20). This was the first demonstration that an intact nicotinic acid works as an acyl primer unit for oligoketide formation in fungal secondary metabolites.[194]

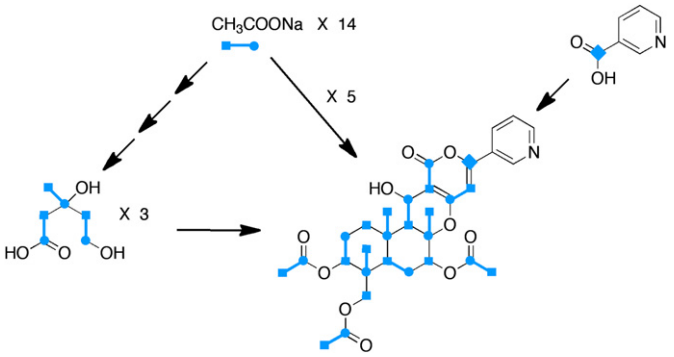
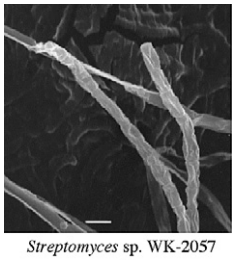
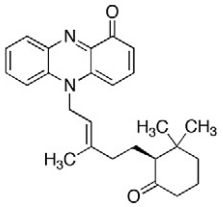


Fig. 20. Biosynthesis of pyripyropene A (**51**).

As described above, increasing our understanding of the biosyntheses and structures of a diverse variety of metabolites allowed us to better speculate on the actual biosynthetic pathway of some of the new microbial metabolites being discovered. For example, phenazinomycin (**113**)[195] is presumed to be biosynthesized through the binding of sesquiterpene to a phenazine skeleton and transforms to the final compound via demethylation or demethylated sesquiterpene binding to phenazine.



Streptomyces sp. WK-2057



Phenazinomycin (**113**)

Looking at the fruits of these biosynthetic studies, we are constantly reminded that microorganisms dexterously synthesize a multitude of metabolites, often by mechanisms which are not yet known or mastered in organic synthesis. For instance, the indolocarbazole core of staurosporine (**16**) is formed in microorganisms by a process yet to be duplicated. Dr. H. Onaka et al.[196] elucidated this using gene cloning as shown in Fig. 21. L-Tryptophan is transformed to the imine form of indole-3-pyruvic acid (IPA imine) (**114**) by *StaO* and two IPA imines are converted to chromopyrrolic acid (**115**) by coupling of *StaD*. The indolocarbazole core (**116**) is formed by *StaP* and *StaC*. These reaction mechanisms at each step may well act as a guide in the development of a comparable organic synthesis reaction.

Looking at compounds possessing multiple pathways for biosynthesis, I think that not only can we expect the discovery of compounds with various combinations but there are also infinite possibilities for obtaining compounds with completely novel structures through genetic manipulation of the producing organism.

4.2. Blocked mutant and hybrid products

As a side-product of our research, we ended up with a variety of 'blocked' mutant microorganisms, in which some of the steps of various biosynthetic pathways had been genetically blocked. We exploited this in biosynthetic studies, centering on macrolide antibiotics, and deduced various biosynthetic pathways by isolating compounds produced by each mutant. As a result, we obtained several novel derivatives and evaluated their biological activities.

We isolated platenolide II (**117**), protylonolide (**118**), and avermectin-related compounds from cultured broths of blocked mutants derived from each producing microorganism of the 16-membered macrolide antibiotics, leucomycin, tylosin, and avermectin. These were used for clarification of biosynthetic pathways and for cloning of genes related to biosyntheses. As described previously, cerulenin inhibits biosynthesis of not only fatty acid but also polyketide. By using the inhibitor, we tried to obtain a hybrid antibiotic and succeeded in getting several novel compounds. In this way, we obtained chimeramycins A (**119**) and B (**120**), both hybrid products, which became the basis for development of an entirely new field of genetically-engineered antibiotics.

The new hybrid antibiotics chimeramycins A and B were produced by adding protylonolide (a lactone corresponding to tylosin aglycone produced by a blocked mutant *Streptomyces fradiae* KA-261[197]) to a culture of spiramycin-producing *Streptomyces ambofaciens* ATCC-15154, grown in the presence of cerulenin to inhibit the biosynthesis of aglycone plantenolide[198] (Fig. 22).

For biosynthesis of chimeramycins, complicated manipulation is required. For example, protylonolide (**118**) is required, an intermediate of tylosin biosynthesis from a mutant of *S. fradiae* KA-

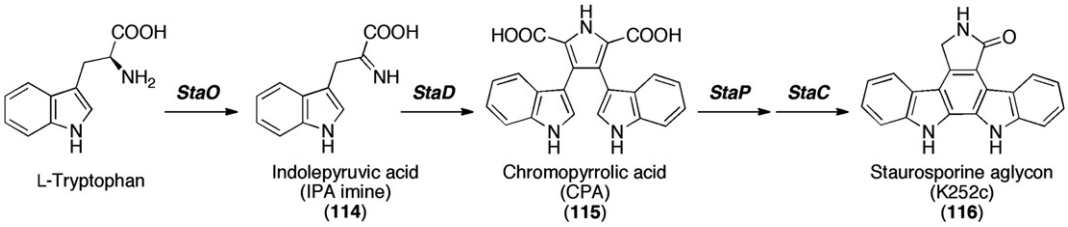


Fig. 21. The formation of indolocarbazole core in the biosynthesis of staurosporine.

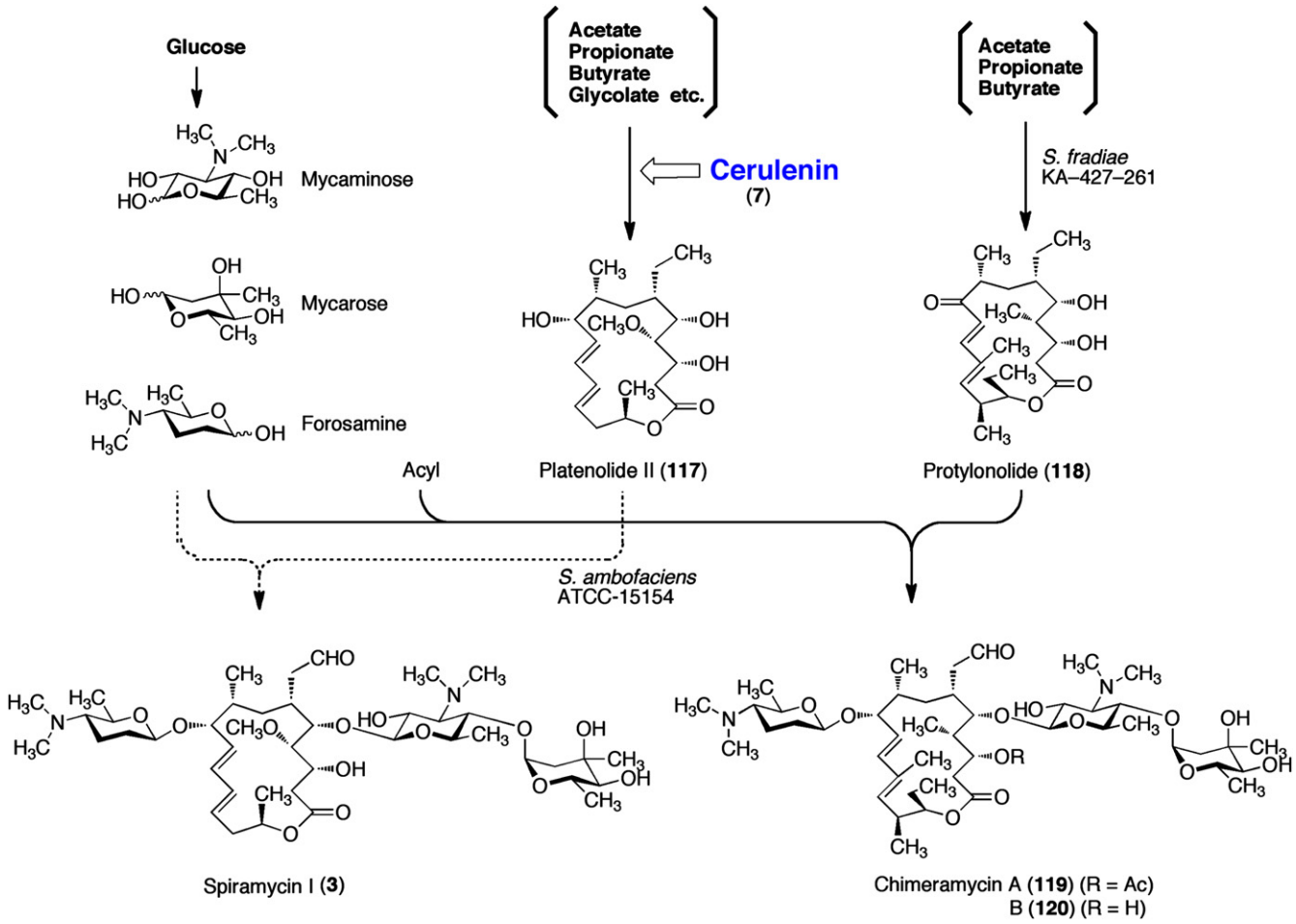
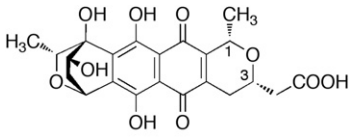


Fig. 22. Hybrid biosynthesis of chimeramycins A (**119**) and B (**120**) by a spiramycin-producing organism, *S. ambofaciens* ATCC-15154.

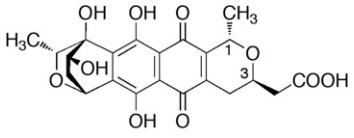
427-261, which is a tylosin producing microorganism. Thus, the first trial to obtain hybrid antibiotics via genetic engineering was done through collaborative research with Prof. D. Hopwood of John Innes Institute, UK, Prof. H. Floss, Univ. of Washington, USA and myself. These two individuals are my good friends as well as being excellent researchers and top-drawer scientists and we have enjoyed a long and fruitful collaboration, which culminated in the generation of two kinds of new genetically-engineered hybrid antibiotics, mederrhodins A (**121**), and B (**122**) and dihydrogranatirhodin (**123**).[199,200] The isolation process for mederrhodins A and B is shown in Fig. 23.

Recombinant plasmid pIJ2315, consisting of an SCP2-based vector plus an *actVA* gene (the step involving hydroxylation at the C-6 position of isochromanequinone in the gene cluster for actinorhodin biosynthesis in *Streptomyces coelicolor* A3), was introduced into the medermycin-producing *Streptomyces* sp. AM7161 to construct a genetically-engineered strain, *Streptomyces* sp. AM7161/pIJ2315. The transformant produced the new hybrid antibiotics identified to be 6-hydroxy-medermycin and 6-hydroxydihydromedermycin, and named mederrhodins A and B, respectively. Using the same method, dihydrogranatirhodin (**123**), which is an epimer at C-3 of dihydrogranaticin (**124**), was manufactured.

Soon thereafter, biosynthetic studies of microbial metabolites involving manipulation at the genetic level quickly burgeoned, leading to the creation of an impressive array of novel compounds.[201–203]



Dihydrogranatirhodin (**123**)



Dihydrogranaticin (**124**)

4.3. Biosynthesis of avermectin; cloning of biosynthetic gene clusters

In 1982, when my student Dr. H. Ikeda received his Doctorate degree in Pharmaceutical Sciences from Kitasato University, I asked Prof. Hopwood, who was a UK-based world leader in the genetic studies of actinomycetes, if there was a possibility for him to accept Dr. Ikeda as a post-doctoral fellow. In those days, I thought that genetic research of actinomycetes would become an important focus, particularly with respect to the production of bioactive microbial metabolites. Fortunately, Prof Hopwood was most accommodating and Dr. Ikeda had an excellent opportunity to forge ahead

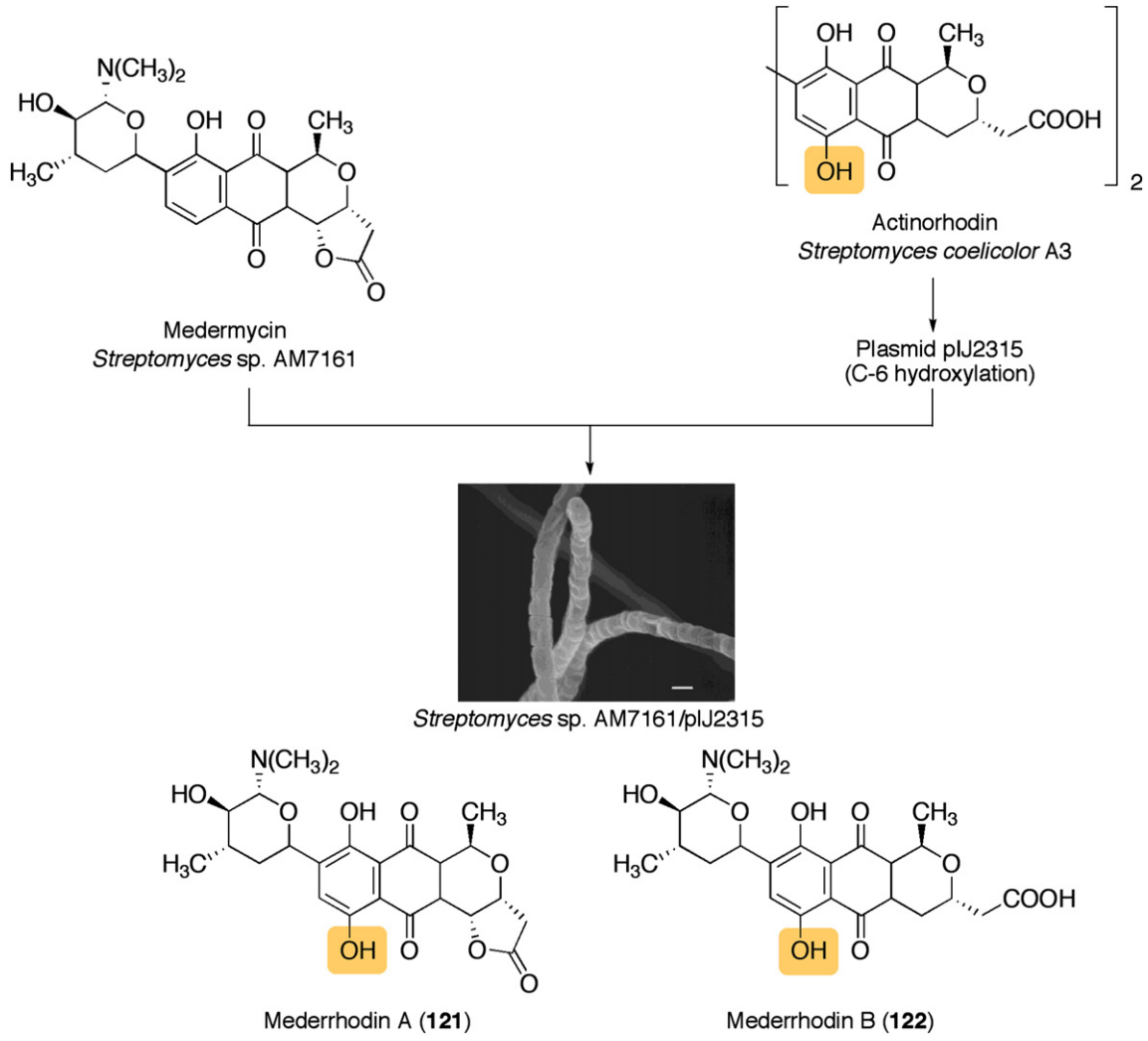


Fig. 23. Production of mederrhodins A (**121**) and B (**122**), the first genetically-engineered antibiotics.

with genomic work on actinomycetes, the outcome of which played a significant role in promoting our future metabolite research (as described in Section 4.2).

Prof. H. Ikeda's team became part of our multidisciplinary research group and their results in terms of mapping of biosynthetic genes, elucidation of biosynthetic pathways and overall genome analysis of the avermectin-producing microorganism, *Streptomyces avermitilis* MA-4680^T (*S. avermectinius* is a junior homotypic synonym of *S. avermitilis*), is described in detail in the next section. Their work allowed us to create mutant organisms in which avermectin biosynthesis was blocked. Thorough stepwise analysis allowed identification of single-point mutations, elucidating the structures of biosynthetic intermediates produced by each mutant (Fig. 24) and identifying their locations in the biosynthetic pathways. Moreover, the information taken from these blocked mutants became the basis for the cloning of gene cluster for avermectin biosynthesis. The results of the biosynthetic studies of avermectin are summarized in Figs. 25 and 26.[204,205]

The gene products believed to be associated with various steps are indicated at respective points in the pathway.

Seventeen genes responsible for avermectin biosynthesis were eventually identified and their functions were analyzed by cloning.[206–209] As shown in Fig. 25, four genes, *ave*A1, *ave*A2, *ave*A3, and *ave*A4, are involved in the biosynthesis of the basic skeleton of the aglycone moiety. AVES1/AVES4, whose synthesis is governed by these four genes, are multifunctional proteins composed of 3973, 6239, 5532, and 4681 amino acids, respectively. There are a total of 12 modules in these four large, multifunctional proteins. The AT (acyltransferase) domain transports acyl groups necessary for acyl-chain elongation, one after another, to the ACP (acylcarrier protein) domain present in each module. The acyl groups are then condensed by the catalytic action of the KS (β-oxoacyl-ACP synthase) domain. The resultant β-oxoactyl-ACP is reduced by the KR (β-oxoacyl-ACP reductase) domain and β-hydroxyacyl-ACP is further dehydrated by the DH (dehydratase) domain. As shown in Fig. 25, these chain elongation reactions and lactonization at the final step by TE (thioesterase) domain form the basic skeleton of lactone and the nacent lactone is further modified by cytochrome P450 (AveE: CYP171A1) and C5-ketoreductase (AveF) to form avermectin aglycones. Through reaction of the *ave*B1-*ave*BVII gene's products, namely AveBII-~AveBIII, L-oleandrose is synthesized from glucose-1-phosphate as TDP-L-oleandrose and linked to the aglycone-lactone, completing avermectin biosynthesis (Fig. 26). The presence of two L-oleandroses has been shown to produce the potent antinematode activity of avermectin. The presence of the hydroxyl group at position 13, which allows the binding of L-oleandrose, is extremely important. The DH domain in module 7 at AVES3 is originally involved in the C13—OH dehydration reaction, but when histidine is substituted for tyrosine in its catalytic active center (consensus motif:

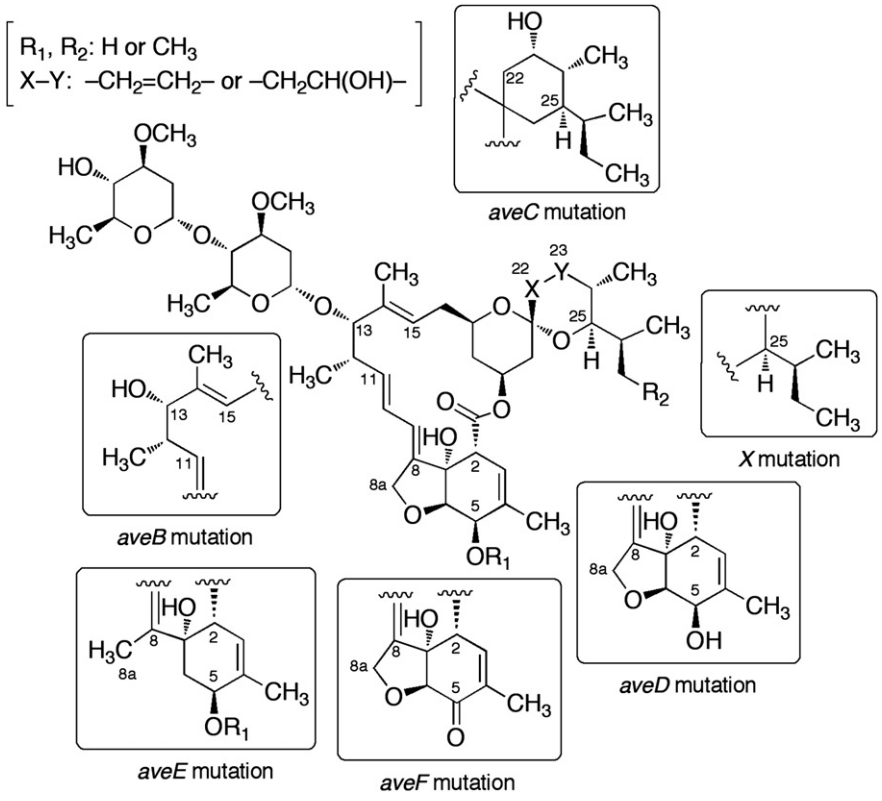


Fig. 24. Blocked mutants of the avermectin producer, *S. avermectinius* (*avermitilis*), and molecular structures of their products.

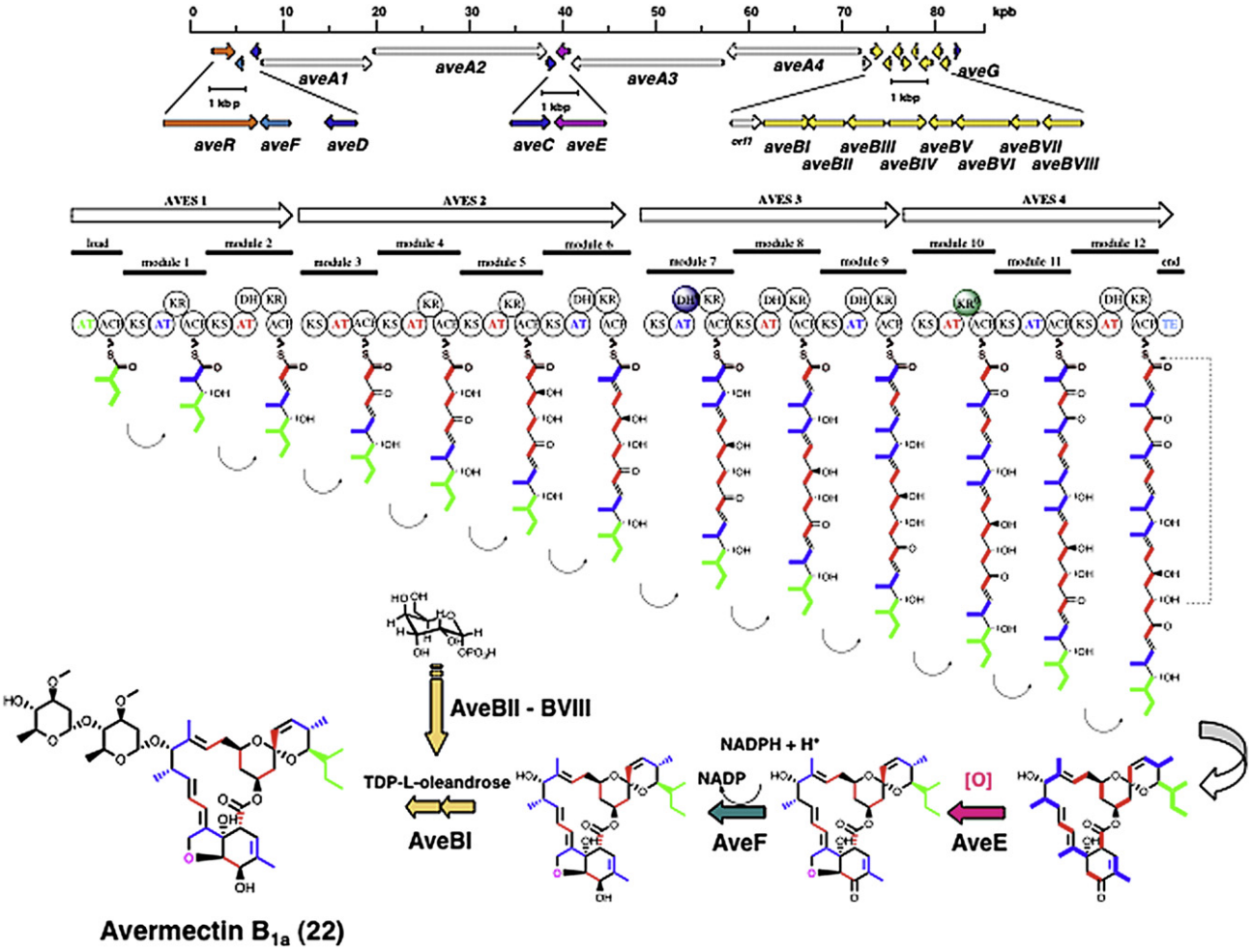


Fig. 25. Gene cluster for avermectin biosynthesis and the overall biosynthesis pathway (Image courtesy of Prof. H. Ikeda).

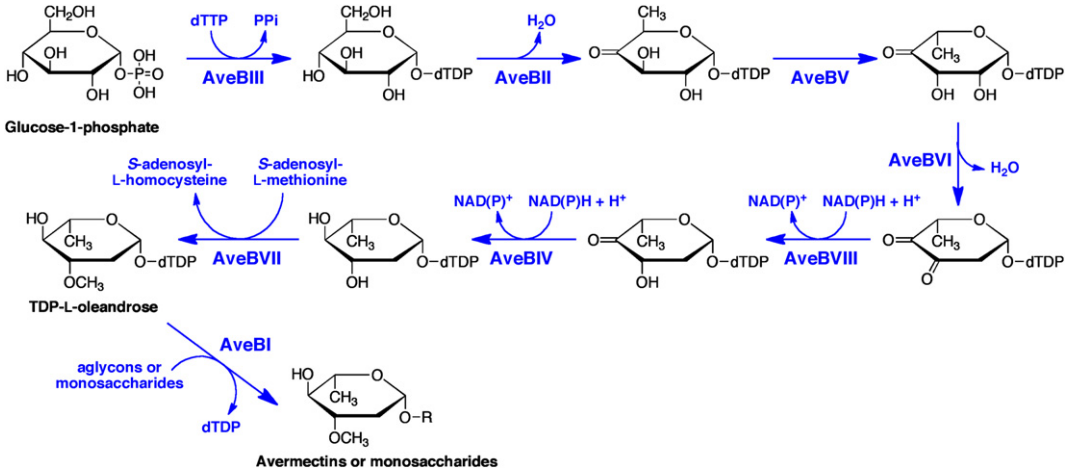


Fig. 26. Scheme for the biosynthesis of L-oleandrose. The gene products believed to be associated with various steps are indicated at respective points in the pathway.

HxxxGxxxxP/S), the domain becomes dysfunctional. Subsequently, biosynthesis progresses, while the hydroxyl group at position 13 remains, forming lactone. This one-point mutation allows the sugar, L-oleandrose, binding and subsequent biosynthesis of avermectin which has superior anthelminic activity compared to metabolites without the sugar moiety, such as milbemycin and nemadectin.[124] Humankind has received enormous benefits from this simple, tiny single-point mutation in the catalytic active center of the DH domain in module 7.

4.4. Genome sequencing of *S. avermectinius* (*avermitilis*) and downstream research

It is well known that microorganisms possess ability to produce various kinds of substances, as exemplified by *Streptomyces griseus*, a streptomycin-producing microorganism that has been used to obtain vitamin B_{12}, as well as antibiotics, since the early-1950s when industrial-level production of antibiotics began using commercial fermentation. At the outset of research, *S. avermitilis* was known to produce oligomycin as well as the compound which was later named avermectin. Oligomycin is similar to avermectin in some of its physico-chemical properties, such as lipophilicity. However, since oligomycin is highly toxic, all avermectin must be produced carefully to ensure that there is no contamination.

Besides the production of avermectin and oligomycin, we were interested in the ability of *S. avermitilis* to generate other secondary metabolites. Therefore, in order to evaluate the overall characteristics of the organism's capabilities, we started intensive genome analysis in 1997. In those days, genome sequence analyses of some bacteria had begun to be reported, such as the work on *Haemophilus influenzae* Rd (KW 20) and *Mycoplasma genitalium* in 1995. As for actinomycetes, a British group led by Prof. D. Hopwood had been making progress in sequencing *Streptomyces coelicolor* A3(2), which has become an important standard in genetic studies of actinomycetes.[210] Although starting 3 years later, we accomplished a full mapping of *S. avermitilis* at almost the same time.[211,212]

The person who played the central role in this new avenue of research was Prof. Ikeda. It was widely believed that the analysis of DNA with high GC content, and that which is specific to actinomycetes, was particularly difficult. Fortunately, we were able to speed up the work, thanks to the participation of Prof. Y. Sakaki of the University of Tokyo (Now president of Toyohashi University of Technology.).

Our group completed analysis of the entire genome (9,025,608 bases) of *S. avermitilis* MA-4680^T (ATCC 31267^T=NRRL 8165^T=NCIMB 12804^T=JCM 5070^T) in 2003. The information obtained, which represented the first genome analysis of an industrially-important actinomycete, provided a major boost for research of secondary metabolites of microorganisms. Fig. 27 shows an outline of the genome, highlighting the gene clusters involved in the biosynthesis of secondary metabolites. We firstly estimated that there were 32 such clusters, and then the latest findings predict that there are 37 clusters involved.[213] The production of oligomycin, along with avermectin, was already known but production of 10 secondary metabolites, including the polyene macrolide, filipine III (*pte*), carotene (*crt*), pentalenolactone (*ptl*), geosimine (*geo*), and nocardamin (*sid*) were all predicted by the genetic analysis, and later confirmed by isolating each metabolite from a fermentation broth of *S. avermitilis*. This created a new research mechanism, whereby production of compounds with specific structures can be predicted by gene analysis and later confirmed through actual production and isolation. The mechanism by which secondary metabolites are produced in *S. avermitilis* has now been fully clarified and work is progressing to engineer the producer microorganism to manufacture yet more potent 'designer' compounds.

Following on from our successful genome-targeted research, we concentrated on elucidation of the concise biosyntheses for various metabolites of actinomycetes, benefitting from the participation of expert researchers such as Prof. D. Cane, Brown University, USA and Prof. H. Oikawa of Hokkaido University in Japan. Recently, we reported the identification and functional analysis of genes controlling the biosynthesis of 2-methylisoborneol (2-MIB) (**125**).[214] In this investigation, it was clarified that geranylpyrophosphate (GPP) is methylated by GPP methyltransferase and cyclized by monoterpene cyclase, ultimately resulting in the generation of 2-MIB.

We have managed to obtain an improved strain of S. *avermitilis*, which contains only 80% of the original genome, produced by removing sequences unnecessary for substance production by the site-specific and homologous recombination technique. Using the genome-minimized strain, the heterologous expression of gene cluster for cephamycin C (**126**) biosynthesis from a *S. clavuligerus* genomic library, was attempted, resulting in astonishing production of bioactive compounds.[215] This is a highly innovative foray in biotechnology, which should provide clues to guide applied research on genetic manipulation and customized culturing systems to facilitate productivity of a range of useful compounds.

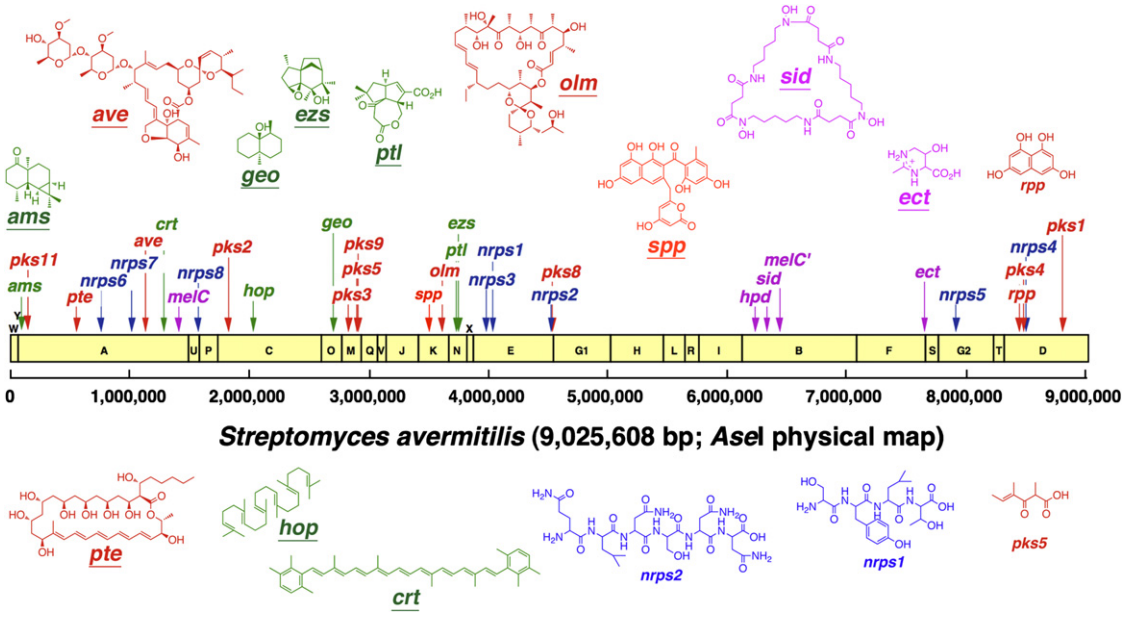
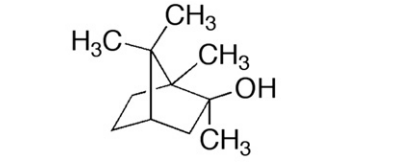
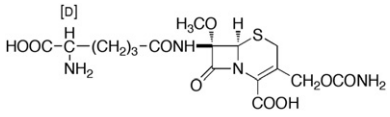


Fig. 27. Chromosome of the avermectin-producing *S. avermectinius* (*avermitilis*) and the distribution of gene clusters involved in the biosynthesis of secondary metabolites (italic abbreviation under each molecular structure indicates the biosynthetic genes whose location is indicated by an arrow on the line chromosome). (—, —, —, —) (Image courtesy of Prof. H. Ikeda).



2-Methylisoborneol (**125**)



Cephamycin C (**126**)

4.5. Production of novel compounds by genetic manipulation

We studied the production of new nemadectin-related compounds having higher potency than the original compound by alteration of a part of the genome in *S. cyaneogriseus* subsp. *noncyanogenus* NRRL 15773, the primary nemadectin-producing microorganism.[216]

The structural difference between nemadectin α (**127**) and avermectin B1a (**22**) (Fig. 28) is basically the presence or absence of an *O*-glycoside bond, which links directly to two oleandroses at position C-13 on the lactone, together with the structure of the-side chain at position C-25. At first, we cloned the gene cluster for nemadectin biosynthesis and compared it with that of avermectin. By manipulating the gene, we introduced L-oleandrosyl-L-oleandroside, in the same manner as with avermectin, at position C-13 of the lactone skeleton of nemadectin. Comparison of domain organization contained in module 7–9 on the AVES of avermectin with those on nemadectin PKS is shown in Fig. 29. In comparing both module 7s, in the case of AVES3, DH does not act, so the hydroxyl residue at position C-13 remains unchanged.

(Heterogenous PKS genes (aveA3-aveA4) were introduced to nemA3-4 mutant using an integrating vector).

However, for NemA3, the C-13 is saturated by action of the ER (enoyl reductase) domain, in addition to DH, forming a methylene residue, and so introduction of the disaccharide becomes impossible. As *nemA*3-*nemA*4 forms an operon, we prepared a strain in which the *nemA*3-*nemA*4 is disrupted via insertion of a viomycin-resistant gene into the *nemA*4 region, subsequently introducing a DNA fragment containing the *aveA*3-*aveA*4 operon via an integration vector which was constructed from *attP* and integrase gene (*int*) of actinophage (Fig. 30). The *attP* works, in efficient site-specific recombination with *attB*, on the chromosome by *int* gene product and integrates *aveA*3, and *aveA*4 into the chromosome. To this mutant ($attB_{\phi C31}$::*aveA*3–*A*4), we also introduced *aveR*, a regulatory gene for avermectin biosynthesis. We were able to create a mutant ($attB_{\phi C31}$::*aveA*3-*A*4, $attB_{TG1}$::*aveR*) capable of producing useful quantities of C-13 hydroxyl nemadectin α (**128**).

Further exploiting the integrated vector system, we introduced a DNA fragment containing the gene cluster of *aveBI-BVIII* covering L-oleandrose biosynthesis and glycosylation, derived from *S. avermitilis* and obtained a C13-*O*-L-oleandsyl-L-oleandrosylnemadectin α (**129**) producing mutant ($attB_{\phi C31}$::*aveA*3-*A*4, $attB_{TG1}$::*aveR*, $attB_{R4}$::*aveBI*∼*BV*III). This compound demonstrated bioactivity against *C. elegans* that was 180-fold higher than that of natural nemadectin α.

I am convinced that further studies in this relatively new line of research are going to provide extremely advantageous results and downstream benefits.

5. Conclusions and future horizons

As described above, I have been fascinated and captivated by microoganisms and their array of metabolites for well over 45 years. My scientific intrigue has been partially sated by the advances made in the understanding of biosynthesis, genetic analyses of producing strains, total synthesis and the structure/activity relationships of the microbial metabolites obtained. This knowledge and appreciation of how microorganisms work, coupled with insights into the science involved, gives us access to the unlimited potential offered by the

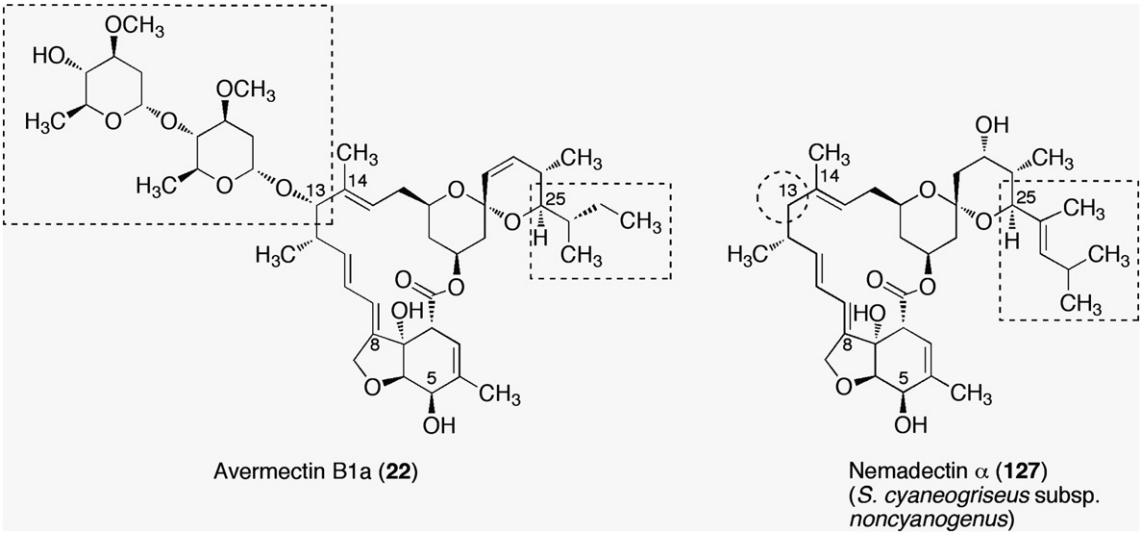


Fig. 28. Strutural comparison of avermectin B_{1a} and nemadectin α.

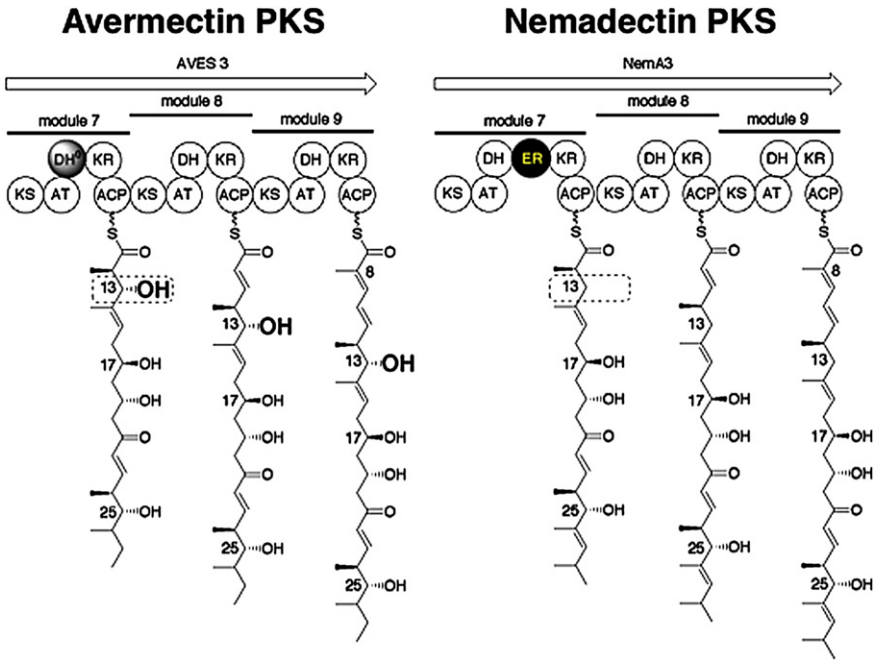
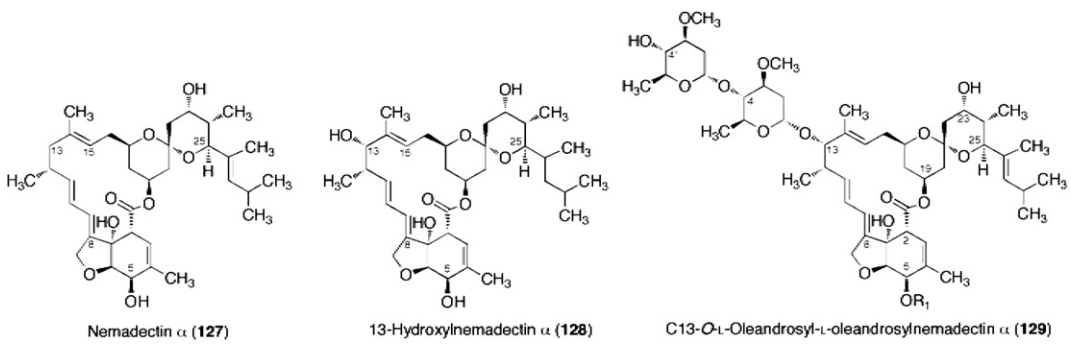


Fig. 29. Domain organizations of AVES3 and NemA3.



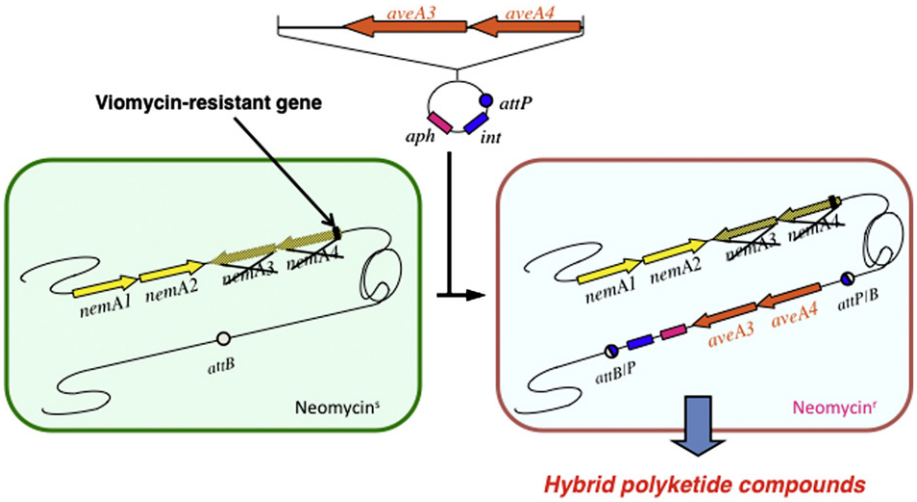


Fig. 30. Construction of a recombinant strain carrying heterogeneous PKS genes. (Heterogenous PKS genes (aveA3-aveA4) were introduced to nemA3-4 mutant using an integrating vector).

artificial production of completely novel compounds, as illustrated in Section 4.5 describing new compounds produced through manipulation of a nemadectin-producing organism.

Until now, we have made special efforts to isolate peculiar and novel microorganisms and to construct a library of microorganisms with the goal of eventually discovering the full range of chemicals that they are capable of producing. Through our searches, we have amassed a library of 25,000 strains, including nine new genera and 31 novel species identified by us (Table 2). Without fail, we have registered and kept in the library all the microorganisms we have unearthed, whether previously known or not. This means that we, in effect, expect the possibility of finding other compounds or bioactivity other than those already reported. This belief has been justified, for example, as described with the case of atpenin A5 (**93**), a compound found initially during the screening for inhibitors of fatty acid metabolism, but later found to be a potent inhibitor of mitochondrial complex II, which is now extensively used as a reagent for research.

I fully expect that, at some point in future, a great deal of as-yet unrecognized bioactivity and biomedical and scientific uses will be identified from further studies of the compounds covered in this account.

We recognize that we may have really just scratched the surface with regard to our discoveries and what remains undiscovered. Consequently, whenever we introduce a new screening system, we re-culture our complete library of microorganisms and re-evaluate both them and our library of compounds. Our goal, was, is, and will remain, the identification of substances that will be of use to improve lives and scientific endeavor worldwide, using Nature as our guide. In addition, we do what we can to ensure that the fruits of our research are used to benefit humankind as quickly as is possible.

Over the past 5 decades, I have been fortunate to have been involved in the discovery of around 440 kinds of novel metabolites. Among them, 25 compounds have been used widely as medicines, agents to improve animal health and husbandry, as agricultural chemicals and as reagents for biochemical research (Table 3). In the process, they have all made significant contributions, big and small, toward the improvement of the health and welfare of men, women and children worldwide, as well as encouraging and accelerating advances in the Life Sciences.

Throughout history, humans have been living with and exploiting microorganisms, as well as being struck down by the variety of diseases that some pathogenic organisms are responsible

Table 2
New microorganisms and their bioactive products discovered by Ōmura's group (2009)

	Microorganisms	Bioactive product
New genus	*Humihabitans oryzae*	
	Humibacillus xanthopallidus	
	Kitasatospora setae	Setamycin (**31**)
	K. cineracea	
	K. griseola	
	K. niigatensis	
	K. phosalacinea	Phosalacine (**34**)
	Longispora albida	Actinohivin (**100**)
	Microterricola viridarii	
	Oryzihumus leptocrescens	
	Patulibacter minatoensis	
	Phytohabitans suffuscus	
	Albophoma yamanashiensis	Terpendoles
New species and new subspecies	*Actinocatenispora sera*	
	Actinoplanes capilaceus	2-Hydroxyethyl-3-methyl-1, 4-naphotoquinone
	Actinomadura corallina	2′-Amino-2′-deoxy adenosine
	Amycolatopsis azurea	Azureomycins
	Arthrobacter humicolus	
	A. oryzae	
	Demequina salsinemoris	
	Kitasatoa griseophaeus	
	Microbacterium aoyamaenses	Quinoline-2-methanol
	M. diminutum	
	M. pumulum	
	M. pygmaeum	
	M. terricola	
	M. echinospora subsp. armeniaca	Clostomicin
	Promicromonospora sukumoe	7-Hydro-8-methylpteroyl-glutamylglutamic acid
	Lentzea aerocolonigenes	Staurosporine (**16**)
	Streptomyces avermectinius	Avermectin B_{1a} (**22**)
	S. candidus subsp. azaticus	OS-3256B
	S. cervinus	Cervinomycin (**32**)
	S. griseoflavus subsp. pyrindicus	Pyrindicin (**15**)
	S. herbaceus	9-D-Arabinofuranosyl-adenine
	S. kagawaensis	Prumycin (**20**)
	S. kitasatoensis	Leucomycin $\mathbf{A_3}$ (**1**)
	S. lactacystinaeus	Lactacystin (**47**)
	S. matensis subsp. vineus	Vineomycin $\mathbf{A_1}$ (**26**)
	S. nodosus subsp. notoensis	Asukamycin
	S. scabrisporus	Hitachimycin
	S. rosa subsp. notoensis	Nanaomycin
	S. subflavus subsp. irumaensis	Irumamycin
	S. taitoensis	Rodiomycin
	Acremonium caerulens	Cerulenin (7)

Table 3
Microbial metabolites[a] used as biochemical reagents discovered at Kitasato[2]

Compound	Activity
Andrastin A (**50**)	Protein farnesyl transferase inhibitor
Atpenin A5 (**93**)	Mitochondrial complex II inhibitor
Cerulenin (**7**)	Fatty acid synthase inhibitor (β-ketoacyl-ACP synthase inhibitor)
Elasnin (**21**)	Human granulocyte elastase inhibitor
Herbimycin A (**28**)	HSP90 inhibitor
Lactacystin (**47**)	Proteasome inhibitor
Macrosphelide A (**64**)	Cell adhesion inhibitor
Madindoline A (**68**)	JAK/STAT signal transduction inhibitor (bind to gpl30)
Oxetin (**35**)	Glutamine synthase inhibitor
Pyripyropene A (**51**)	Acyl-CoA: cholesterol acetyltransferase 2 inhibitor
Setamycin (**31**)	Vacuolar-type H^+-ATPase inhibitor
Staurosporine (**16**)	Protein kinase inhibitor
Arisugacin A (**59**)	Acetylcholinesterase inhibitor
Triacsin C (**41**)	Long chain acyl-CoA synthetase inhibitor
Vineomycin A_1 (**26**)	Collagen prolyl hydroxylase inhibitor

[a] Some key compounds described in this account are listed in this table.

for. Over time, societies everywhere have benefited from basic scientific techniques involving microorganisms, such as fermentation. It cannot be denied that microbial products, especially those with unique or complex structures, have played a major role in stimulating the design of new methods of chemical synthesis and in the evolution of the strategy and tactics of organic synthesis. For some chemists, the attractiveness of many substances as targets for synthesis is their bioactivity. For others, the structural challenge is the incentive. Among the compounds discovered by us, some 100 compounds have become the target for syntheses in organic chemistry, with some 200 or more research groups involved in total synthesis research, meaning that our compounds have also contributed markedly to the progressive development of organic chemistry.

In order to get a better appreciation of how the compounds found by us have been used, and to help evaluate their global impact, we examined all international scientific publications during the period 1995–2009 to determine those which cited the following five microbial metabolites; cerulenin, staurosporine, setamycin, avermectin/ivermectin, and lactacystin. The cumulative total was 17,531, indicating that these handful of compounds alone generated an average of 1100 or so publications each year.[217]

As science advances and our knowledge improves, it is clear to me that the elucidation of suitable targets for medicines, and our expectations for finding remedies to treat both known and as-yet unknown diseases and conditions, will not only improve but also accelerate. Genomic mapping and identification of lead compounds have progressed significantly since the turn of the century, as evidenced by the mapping of the human genome. I believe that naturally-occurring microbial metabolites offer unmatched promise in this respect, but the introduction of novel screening methods will be key to achieving optimal results. Thus, success will be restricted by our vision and our innovation—or lack of it. Fortunately, we have access to some of the innovation we need through genetic engineering[199,216] and the number of non-natural compounds obtained is increasing rapidly as a result. As mentioned above, research is also expected to develop substantially based on the findings of biosynthetic studies and from the investigation of naturally-occurring substances that boast hitherto unseen structures.

Throughout my career, I have been keen to enter into collaborative work of a collegiate and interdisciplinary nature. In many cases, we have shared samples obtained by fermentation or chemical synthesis, provided to colleagues particular microorganisms and biosynthetic genes, and broadcast information on the evaluation of biological activities, and so on. We have worked alongside specialized researchers in fields, such as biochemistry, molecular biology, clinical medicine, social sciences, education, training, and communication. My approach has always been influenced by the tenet 'One encounter, one chance' (一期一会 ichigo-ichie). To me, this means that in life, when an opportunity presents itself, we must seize it quickly because it may be unique. In addition, the shorter that special 'moment', the faster and stronger any bond of the heart will be.

From the moment I start any collaboration until the time it is finished, I always hold any partner in the highest and most respectful esteem, in the spirit that the collaboration will occur but once in our lives. Through my various collaborations, I have been privileged and humbled to have been able to diffuse and gain a great deal of knowledge and understanding, as well as being lucky enough to establish many deep and long-lasting friendships worldwide.

I have also been fortunate in having had some wonderful teachers, role models and mentors, the kind of individuals who remain a crucial element in the success of any scientist—even today. Of the younger generation who I have taught or worked alongside in my laboratory, I am delighted that 27 have risen in the fields of research and education and now hold senior professorial positions in various universities. It gives me great personal satisfaction to know that these individuals will become future leaders and will likely maintain the traditions and spirit of scientific research that have served so many so well in the past. I can rest assured that the future of natural-product chemistry is being placed in safe hands.

I would like to finish this personal essay with a revisiting of 'The Laws of Applied Microbiology', first postulated by the late Prof. David Perlman, who was a great scientist and an accomplished Applied Microbiologist, and who I remember with affection.[218] They exquisitely and succinctly encapsulate my approach to microbial natural-product research.

The Laws of Applied Microbiology
(according to Prof. D. Perlman (1921~1980))

1. The microorganism is {always right / your friend / a sensitive partner}
2. There are no stupid microorganisms
3. Microorganisms {can / will} do anything
4. Microorganisms are {smarter / wiser / more energetic} than {chemists / engineers / etc.}
5. If you take care of your microbial friends, they will take care of your future (and will live happily ever after)

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I am also extremely cognizant of the fact that the development, direction, and success of my research work has been heavily

influenced by a large number of individuals outside of my research team, both national, and international. I owe a great deal of thanks to this veritable army of highly-talented and visionary friends and colleagues.

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